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Metagenomic Characterization And Biochemical Analysis of Cellulose-Degrading Bacterial Communities from Sheep Rumen, Termite Hindgut, Decaying Plant Materials, and Soil

By

Alonzo B. Anderson

A thesis submitted to the Faculty of The School of Graduate Studies, Research, and Outreach of VIRGINIA STATE UNIVERSITY in partial fulfillment of the requirements for the degree of Master of Science in the College of Natural and Health Sciences

Virginia State University
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TABLE OF CONTENTS

LIST OF FIGURES……..…….……..iii-iv

LIST OF TABLES……...……..…..…..v

ABSTRACT…….……...…vi

CHAPTER I. INTRODUCTION…….…1-12

CHAPTER II. METHODOLOGY…….……...……13-21

CHAPTER III. RESULTS…….…..…….……22-66

CHAPTER IV. DISCUSSION and CONCLUSION.…….…..……67-71

REFERENCES……..….72-74

LIST OF FIGURES

Figure Number	Description	Page Number
1	Flow chart outlining the approach for characterizing the cellulose-degrading bacteria in sheep rumen, termite hindgut, soil, twig, and decaying plant and their biochemical activities.	12
2	Relative abundance of bacteria at the phylum level from uncultured termite hindgut #1 sample and the selective culture of leaf sample #1 and the two twig samples	29
3	Relative abundance of Proteobacteria at the class level from uncultured termite hindgut #1 sample and the selective culture of leaf sample #1 and the two twig samples	30
4	Relative abundance of Firmicutes at the class level from uncultured termite hindgut sample #1 and the selective culture of leaf sample #1 and the two twig samples	31
5	Relative abundance of Bacteroidetes at the class level from uncultured termite hindgut #1 and the selective culture of leaf sample #1 and the two twig samples	32
6	Alpha Diversity measures of the uncultured termite hindgut sample #1 and the selective culture of leaf sample #1 and the two twig samples	33
7	Relative abundance of Bacteria at the phylum level from the selective cultures of ten other samples	34
8	Relative abundance of Proteobacteria at the class level from the selective cultures of ten other samples	35
9	Relative abundance of Firmicutes at the class level from the selective cultures of ten other samples	36
10	Relative abundance of Bacteroidetes at the class level from the selective cultures of ten other samples	37
11	Alpha Diversity measures from the selective cultures of ten other samples	38
12	Termite hindgut microbiome characterized from the phylum to the genus level	39

13	Decaying leaf sample #1 microbiome characterized from the phylum to the genus level	40
14	Plant twig sample #1 microbiome characterized from the phylum to the genus level	41
15	Plant twig sample #2 microbiome characterized from the phylum to the genus level	42
16	Sheep Rumen #1 0.22 micrometer filtrate microbiome characterized from the phylum to the genus level	43
17	Rumen #1 combined wash-off microbiome characterized from the phylum to the genus level	44
18	Sheep Rumen #1 unfiltered microbiome characterized from the phylum to the genus level	45
19	Sheep Rumen #2 0.22 micrometer filtrate microbiome characterized from the phylum to the genus level	46
20	Rumen #2 combined wash-off microbiome characterized from the phylum to the genus level	47
21	Sheep rumen #2 unfiltered microbiome characterized from the phylum to the genus level	48
22	Microbiome of the termite colony from the Appomattox River bank characterized from the phylum to the genus level	49
23	Microbiome of the termite colon from the east woods of VSU characterized from the phylum to the genus level	50
24	Soil sample microbiome characterized from the phylum to the genus level	51
25	Decaying plant microbiome characterized from the phylum to the genus level	52
26	Standard curve for cellulose estimation	55
27	Standard curve for glucose estimation	56

LIST OF TABLES

Table Number	Description	Page Number
1	Description of sample collections	14
2	Measurement of cell concentration at optical density 600 nanometers	53
3	Total number of bacterial nucleotide sequence reads per sample	53
4	Cellulose standards	54
5	Cellulose measurement	54
6	Glucose standards	56
7	Glucose measurement	56
8	Summary of biochemical analysis	57
9	Comprehensive listing of cellulolytic bacteria	58-66

ABSTRACT

Metagenomic Characterization and Biochemical Analysis of Cellulose-Degrading Bacterial Communities from Sheep Rumen, Termite Hindgut, Decaying Plant Materials, and Soil

By Alonzo B. Anderson,

Under the direction of Dr. Xianfa Xie

In an effort to develop an affordable, clean, and renewable next-generation biofuel technology, this project takes a metagenomic approach to characterize cellulose-degrading bacteria from various samples, including termite gut, sheep rumen, soil, and decaying plant materials. Using selective media culture with cellulose and 16S rRNA, gene sequences, cellulose-degrading bacteria have been identified from each sample from the phylum to the genus level. The samples vary significantly in the diversity of cellulose-degrading bacteria, with each of the sheep rumen and termite gut samples containing the highest diversity while the partially decaying plant meterials the lowest diversity. Interestingly, the two sheep rumen samples differ significantly in their composition of cellulose-degrading bacteria. However, the two termite samples are quite similar with respect to cellulose-degrading bacterial composition, and the termite sample and soil sample collected at the same site are very similar in their cellulolytic bacteria composition. Overall the cellulolytic bacterial community is dominated by three phyla: Proteobacteria, Firmicutes, and Bacteroidetes. Furthermore, biochemical analyses were conducted to assess the cellulolytic activities of the cellulose-degrading bacterial community in each sample, which shows a generally high efficiency in converting cellulose into glucose

CHAPTER I

INTRODUCTION

The Need for Bioethanol

The idea that more abundant, cleaner, and less expensive fuel sources could one day supply the world's energy demands is no longer a far-fetched idea. As economic, political, and social conflicts arise in response to foreign oil dependency, there is an immediate need for localized long-term renewable energy sources. Currently, fossil fuels account for 84% of the country's energy consumption (The World Bank 2015), which presents harmful health and environmental consequences alike.

Fossil Fuels are hydrocarbons consisting of oil, coal, and natural gas formed from the remains of dead plants and animals. The origin of fossil fuels dates back millions of years ago during the Carboniferous Period. The combustion of fossil fuels result in harmful emissions of greenhouse gases such as carbon dioxide and monoxide being released into the earth's atmosphere. The emission of these gases contributes to the warming of the earth's core potentially causing higher temperatures, sea levels to rise, an increase in wild fires, and onset of acid rain. Could there be a solution to this prevailing crisis of non-renewable energy consumption, the answer could very well lie in the production of biofuel.

Biofuel is a promising solution to combat the depletion of the world's non-renewable fossil fuels. Today, bioethanol is the most widely used source of biofuel.

Ethanol is a renewable energy, which by definition means it utilizes natural energy that will never run out and can be reused (Kerr 1998). Currently, the United States produces two types of ethanol, fermentation ethanol and synthetic ethanol (Mielenz 2011). Synthetic ethanol is produced from ethylene, a petroleum by-product, and is used mainly in industrial. Fermentation ethanol (bioethanol) is produced from corn or other biomass feedstocks and is the most common type of ethanol produced, accounting for more than 90% of all ethanol production (Mielenz 2011). The combustion of bioethanol results in clean emission of heat, steam, and most importantly carbon dioxide.

Next-Generation Bioethanol Production Technology

Bioethanol has become increasingly popular due to its potential implications as an alternative fuel. Since ethanol acts as an oxidizing agent, blending it with gasoline that emits lower quantities of carbon monoxide, nitrogen oxide, and hydrocarbon after combustion can be extremely advantageous (Kumar 2009). Traditionally, feedstocks such as grasses, sugarcane, and corn have been used for bioethanol production, but due to the social and economical cost of bioethanol production from these crops, alternatives have recently been explored (Kumar 2009). Today, there is much research surrounding lignocellulose biomass as an alternative due to its abundance on earth. All green plants produce cellulose, making it the most abundant biological molecule in existence. Plants use cellulose as strengthening material, much like a skeleton that allows plants to stand

upright and grow toward the sun, withstand environmental stresses, and to defend against herbivores. Infamously known as the most abundant organic compound on earth, cellulose and hemicellulose have the greatest potential to resolve both the energetic and environmental demands of bioenergy. Cellulosic biomass from trees, shrubs, grasses, and other plant wastes provide abundant materials for next-generation bioethanol production.

The process to convert lignocellulosic biomass into bioethanol involves four major steps: pretreatment, hydrolysis, fermentation, and separation. Initially, all feedstocks must be pretreated to disrupt the naturally resistant carbohydrate-lignin shield that limits the accessibility of enzymes to cellulose and hemicellulose and disrupt the crystalline structure and reduce the degree of polymerization of cellulose (Zheng 2009). Of all the processing steps, pretreatment is the most expensive; with technological improvement in this step the bioethanol production cost can be dramatically reduced (Zheng 2009). Once lignocellulosic plant materials have been pretreated, they must then be hydrolyzed, during which complex polysaccharides in the plant biomass are broken down into simple sugars (glucose). After the cellulose of the feedstocks has been broken down into simple sugars, microorganisms are used to ferment the glucose molecules into ethanol. Yeast, typically baker's yeast, or bacteria are added to the biomass material, which feed on the sugars to produce the fermented ethanol and carbon dioxide. After the fermentation process is complete, the ethanol produced is separated from the fermentation broth through the use of distillation. Distillation separates this mixture by

boiling the liquid mixture of water and ethanol. Ethanol vapors are separated from the liquid portion because ethanol has a lower boiling point compared to water. Enzymatic processing of cellulosic bacteria strains remains the best chance to affordable processing and production of this material (Sangkhara 2011).

Cellulose is a linear polysaccharide polymer with many glucose subunits. Hydrolysis can be achieved in one of two ways. The first is through the use of acids, typically surphuric acid. The second and more favored is through the use of cellulases, which are enzymes breaking cellulose down into simple sugars. Contrary to the use of acids, these enzymes have no by-product or disposal complications and thus tend to be favored. Cellulases are a complex system of enzymes, which usually consists of three types of enzymes, namely endo-1, 4- β -D-glucanase, exo-1, 4- β -D-glucanase, and β -glucosidase. These three types of enzymes work together to hydrolyze cellulose into glucose.

Methods to increase the activity of cellulases and simultaneously decrease production costs have attracted the attention of both scientists and industry (Bayer *et al.* 2007). The major challenge to the above seemingly ideal enzymatic hydrolysis approach is the production of cellulases, which may be costly (Zheng 2009). However, through long history of evolution, many microbes on earth have evolved the capability to produce these enzymes and use them to break down lignocellulosic materials to meet their own energy need. Cellulases are found in a variety of bacteria and fungi, which can be used

for cellulose hydrolysis. However, the cellulose-degrading microorganisms still need to be characterized from the various sources they could be found in.

Existing Studies of Cellulose-Degrading Bacteria in Various Samples

Termite Gut

The microbial community of the termite gut has been a focus in the study of cellulose digestion due to the numerous potential applications in biofuel production (Tartar 2009). Termites are one of the most important soil insects that efficiently decompose lignocelluloses with the aid of their associated microbial symbionts into simple sugars, which later can be fermented to ethanol using yeasts (Upadhyaya 2012). This group of insects contain a community of symbiotic microorganisms in their hindguts including bacteria, archaea, and eukarya that are necessary to break down raw plant material (Li *et al.* 2003). These microorganisms make it possible for termites to process nutrients and energy from wood, while the termite host provides an oxygen free environment to thrive. Termites use their gut microbes to break down cellulose into simple sugars that can be used as an energy source.

Termite comprises of a complex group of diverse species, divided into higher and lower termites. Lower termites harbor a dense population of prokaryotes and protist (single celled eukaryotes) in their gut (Ohkuma 2003). Higher termites comprise only one apical family (Termitidae) but more than three quarters of all termite species (Ohkuma

2003). While they also harbor a dense and diverse array of prokaryotes, higher termites lack protists (Ohkuma 2003). The termite's intestinal tract consists of three main components, the foregut (including the crop and muscular gizzard), midgut, and hindgut. Amongst the three, the midgut and the hindgut are the two independent cellulose-digesting systems (Fujita 2009). In lower termites, much of the cellulolytic activity is found in the hindgut (Upadhyaya 2012). Digestion occurs due to cellulases that are manufactured in the gut that catalyzes the hydrolysis of cellulose. In the midgut, cellulose digestion is carried out by endogenous cellulases, while the hindgut uses cellulases from symbiotic bacteria to break down the cell walls of plant material (Fujita 2009). The hindgut of wood-feeding termites is the largest of the intestinal components and the major site for the absorption and digestion of nutrients, hence its importance to researchers in the study of cellulases. More than 200 species make up the microbial community, converting 95% of cellulose into simple sugars within 24 hours (DOE Genomics 2007).

The study by Upadhyaya *et al.* (2012) based on morphology, biochemical, and molecular analysis have categorized the gut bacteria community into three specific genus groups: Cellulomonas, Enterobacter and Citrobacter. In another study, different cellulose-degrading bacteria have been characterized in a wood-feeding termite native to Asia also using biochemical, morphology, and molecular techniques (Pourramezan 2012). This study revealed isolates of bacteria at the genus level belonging to Pseudomonas with

more than 90% confidence, and Acinetobacter, with more than 80% confidence all belonging to the Proteobacteria phylum. Among these, isolates that were able to grow in aerobic and anaerobic conditions were classified as facultative anaerobic organisms. Further analysis show three isolates identified as gram-positive and thriving only in aerobic conditions. Their analysis produced two other isolates that belong to the genus Bacillaceae with more than 80% confidence. The gram-positive isolates were identified as Staphylococcus with more than 80% confidence. Cellulases from the genus Bacillus were found to be commercially most valuable due to its stability in high temperatures and CMCase activity over a broad range of pH (Pourramezan 2012).

Rumen

Ruminants are a group of herbivores that have a highly specialized digestive system necessary for the digestion and breakdown of grasses into nutrients. This system includes a four-chambered stomach that includes the abomasum (true stomach) and three supporting compartments necessary for digestion to occur, the reticulum, rumen, and omasum. Included in this group of mammals are cattle, goats, sheep, giraffes, yaks, deer, camels, llamas, and antelopes. Their diets consist of cellulose rich feedstocks that must be broken down to extract nutrients. Ruminants along with other vertebrates cannot naturally produce the enzyme cellulases, so they depend on the microbial community of the stomach to dissociate the cellulose. This community consists of microorganisms such as bacteria, protozoa, and fungi that break down feedstock through fermentation (Correa

2007). The products of this metabolic process are used mutually by the ruminant for energy consumption and by the microbial community for cell growth and reproduction. Another study identified three cellulose-degrading bacterial species: *Bacteroides succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Russell 2009). However, a metagenomic study in cow has failed to identify individual microorganismal groups in the rumen (Hess *et al.* 2011)

Decaying plant and Soil Microbiota

The complex process of cellulose degradation is carried out by a series of enzymes necessary for this process to occur. In soils, decomposition can be accomplished directly through the activities of aerobic and anaerobic bacteria capable of degrading cellulose to glucose (Imshenetsky 1968). Cellulose-fermenting microorganisms in anaerobic environments tend to degrade larger quantities of cellulose (Leschine 1995). Cellulose-degrading bacteria obtain carbon from the decomposition of organic matter. Since soil is a huge reservoir of carbon, the anaerobic degradation of soil organic matter plays an important role in the global cycling of carbon (Leschine 1995). Previous microbial analysis of soil collected from Phatthalung, Thailand has revealed 5 strains of cellulose-degrading bacteria. Based on the 16S rRNA nucleotide sequence, all the 5 cellulose-degrading enzymes were identified as *Cellulomonas sp* (Sangkhara 2011).

Metagenomic Study of Bacterial Community Using 16S rRNA Gene

This project takes a metagenomic approach to characterize the bacteria community of sheep rumen, termite guts, decaying plant, and soil, with particular focus on identifying cellulose-degrading bacteria to help develop next-generation bioethanol production technology.

The collective genomes of microbes have been termed the 'metagenome' and these environmental studies are metagenomic studies or shortly metagenomics (Handelsman *et al.*, 1998). It involves extracting DNA directly from an environmental sample and then studying the DNA sample. Metagenomic DNA is complex since it is a pool of genomes from many different organisms, making its analysis challenging (Handelsman 2007). The study of microbial diversity based on 16S rRNA studies termed phylogenetics, is based on analysis of the highly conserved 16S rRNA gene, which accounts for only 0.05% of the microbial genome (Steele & Streit 2005). The synthesis of universal PCR primers amplifies rRNA genes from the DNA of all organisms present in a sample and, when followed by cloning and sequencing, can generate a huge quantity of environmental data regarding sample diversity (Streit & Schmitz 2004). Prokaryotic physiological and metabolic diversity cannot be assessed in its entirety using 16S rRNA gene analysis alone (Steele 2005), and it is recommended to use in conjunction with other investigations. Expression of specific traits can also be sought, such as enzyme activity, glucose concentration, or antibiotic production (Kang *et al.* 2009)

The 16S rRNA gene is a section of the prokaryotic DNA found in all bacteria and

archaea. This gene codes for a rRNA (ribosomal Ribonucleic Acid) molecule, which is a major component of the ribosome. This gene sequence provides genus and species level identification for isolates that do not fit any recognized biochemical profiles (Janda 2007). The 16S rRNA gene has been by far the most commonly used genetic marker for metagenomic studies due to the following reasons (i) it is present in all bacteria; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large and variable enough for bioinformatic analysis (Patel *et al.* 2001). Consequently, the 16S rRNA gene represents the most important target of study in bacterial evolution and ecology, including the determination of phylogenetic relationships among taxa, the exploration of bacterial diversity in the environment and the quantification of the relative abundance of taxa of various ranks (Větrovský 2013).

Research Plan and Objectives

For comparative analysis, samples were cultured in two types of media: Luria-Bertani broth (LB) and a selective cellulose media. To determine the specific microbiome, each sample was initially cultured in LB media. Analysis of this culture will provide the overall composition of bacteria in each of the samples. Since cellulolytic bacteria is the specific interest for this project, the second set of samples were cultured in selective medium containing cellulose only. This media will serve as a second round of

selection. Only bacteria that can grow in the presence cellulose will remain and can potentially be used to break down cellulose into simple sugars during the bioethanol process. From these cultures, the genomic DNA was extracted. Next, the V1-V3 region of the 16s rRNA gene was amplified using PCR. Once purified, the samples were sequenced using Roche 454 sequencing technology. Sequences were analyzed to characterize the diversity of bacteria from different samples. For cellulose selective cultures biochemical assays were further conducted to analyze the remaining cellulose and glucose in each culture. The overall work flow is described in Figure 1.

Successful completion of this project will:

i. Identify the specific cellulose-degrading microbes in soil, rumen, termite, and decaying plant samples.
ii. Quantify the relative abundance of cellulose-degrading bacteria in soil, rumen, termite, and decaying plant collections.
iii. Analyze the cellulolytic activities of the bacteria from soil, rumen, termite, and decaying plant samples.

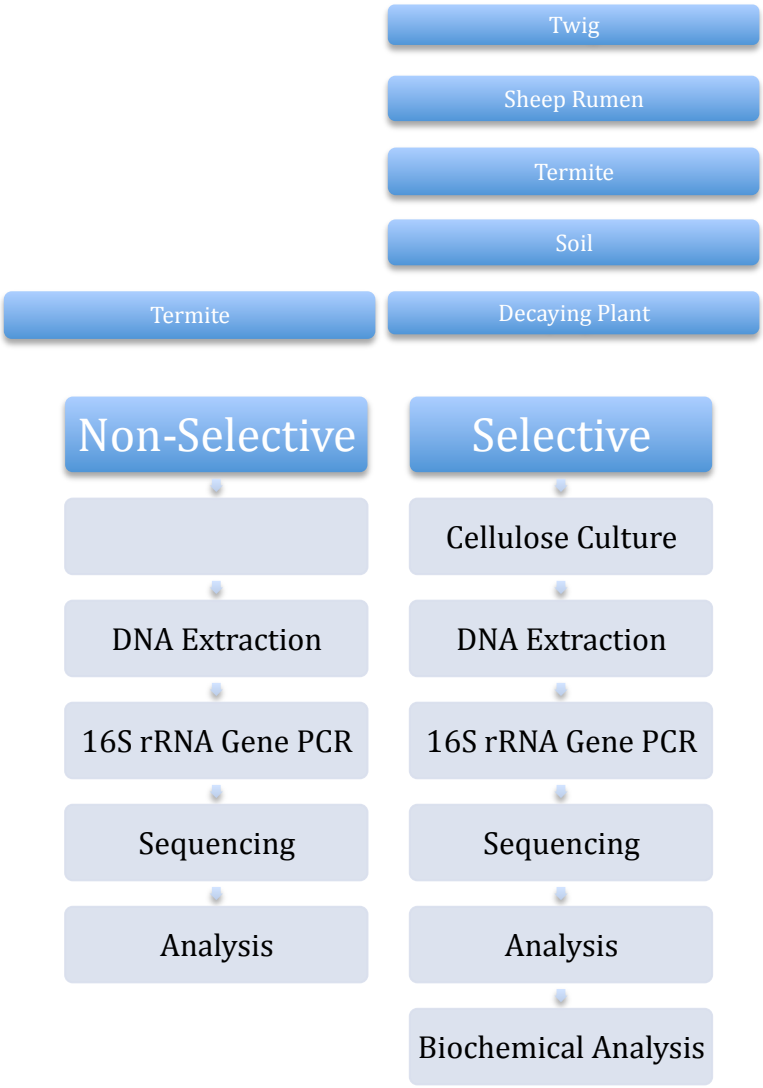

Figure 1. Flow chart outlining the approach for characterizing the cellulose-degrading bacteria in sheep rumen, termite hindgut, soil, twig, and decaying plant and their biochemical activities.

CHAPTER II

METHODOLOGY

Sample Collection

The rumen samples from two individual sheep were collected from a private slaughtering facility at Harrisonburg, Virginia. The two termite colonies were collected from the bank of the Appomattox River to the south of the Virginia State University and the forested area on the east side of the Virginia State University campus, respectively. Three soil samples were also collected from the forested area on the east side of the Virginia State University campus, one of them at the same site where a termite colony was sampled. The decaying leaf was collected at the Bear Creek State Park with permission.

After the collection, each of the two sheep rumen samples was filtered at different levels from 70 microns to 0.22 microns. The 0.22 micron filtrate, the combined filtrates, and the unfiltrated rumen liquid were used as separate samples for subsequent metagenomic and biochemical analyses. The sample IDs, the sample types, and collection locations are described in detail in Table 1.

Selective Cell Culture

Each sample was initially cultured in 10ml of LB media that was prepared using trypton (10 grams), yeast extract (5 grams), NaCl (10 grams), and 500 milliliters of ddH_2O. All samples were incubated at 35°C in a shaker at 180rpm for 24-72 hours, until colony growth was visible.

Table 1 Description of Sample Collections

ID	Source	Site
C1_1:100	Termite Hindgut, uncultured	East Woods, Virginia State University Campus
S5_AA	Decaying Leaf #1	Bear Creek State Park, VA
S6_AA	Twig #1	Bear Creek State Park, VA
S7_AA	Twig #2	Bear Creek State Park, VA
C1	Sheep Rumen #1 0.22 micrometer filtrate	Private facility at Harrisonburg, VA
C2	Sheep Rumen #2 0.22 micrometer filtrate	Private facility at Harrisonburg, VA
C3	Rumen #1 combined wash-off	Private facility at Harrisonburg, VA
C4	Rumen #2 combined wash-off	Private facility at Harrisonburg, VA
C5	Rumen #1 unfiltered	Private facility at Harrisonburg, VA
C6	Decaying Leaf #2	Bear Creek State Park, VA
C7	Soil Collection	East Woods, Virginia State University Campus
C8	Termite Hindgut	Appomattox River, to the south of Virginia State University Campus
C9	Rumen #2 unfiltered	Private facility at Harrisonburg, VA
C10	Termite Hindgut	East Woods, Virginia State University Campus

One ml of each LB culture was used to inoculate 10ml of selective cellulose medium, which consisted of cellulose (2 grams), $NaNO_3$ (1.25 grams), KH_2PO_4 (1 gram), $MgSO_4$ (0.1 gram), NaCl (0.1 gram), and 500 milliliters of ddH2O. The inoculated cellulose selective media were cultured for 10 days at 35°C and 260rpm. The cell density in each culture tube was measured by the absorbance at 600 nanometers from 1 milliliter of culture using SmartSpec Plus spectrometer.

Genomic DNA Extraction

For the nonselective culture, the hindguts from 30 termites were dissected and collected into the same 1.5 milliliters microcentrifuge tube with buffer, which was then used to extract the genomic DNA using the same method as described below.

For DNA extraction from selective cultures, 1.5 milliliters of the culture for each sample was centrifuged for 2 minutes until a compact pellet formed at the bottom of a 1.7 milliliters centrifuge tube. After the supernatant was removed, the pellet was resuspended in 570 microliters of 1X TE buffer. Next, 30 microliters of 10% Sodium dodecyl sulfate (SDS) was used to lyse the cells to release the genomic DNA. The SDS detergent causes the cell membrane to break down by emulsifying the lipids and proteins of the cell and disrupting the polar interactions that hold the cell membrane together. The detergent then forms complexes with these lipids and proteins, causing them to precipitate out of solution. The mixture was incubated for 60 minutes at 65°C. 1/10 volume of 3.3M NaOAc was added to the mixture to get a final concentration of 0.3M NaOAc, then incubated at -20°C for 30 minutes. After the incubation, the samples were centrifuged for 15 minutes at 14,000 rpm and 4°C to collect a protein and cellular debris pellet at the

bottom of each tube. An equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added and well mixed with the other reagents by inverting the tube several times. The tube was incubated on ice for 5 minutes and then centrifuged at 14000rpm at 4°C for 30 minutes. Three layers emerged after the centrifugation. The top layer was an aqueous, polar phase supposedly containing nucleic acids and water, the bottom layer contains mostly of phenol, and a very thin phase between the two layers contains denatured proteins and other cell components. The top layer (supernatant) was then carefully transferred into a sterile 1.7 microliters microcentrifuge tube. Then, 0.6 volume of 100% isopropanol was added, which was then centrifuged for 30 minutes at maximum speed and 4°C. Isopropanol induces a structural change in the DNA that causes them to aggregate and precipitate out of the solution. The remaining supernatant was removed and the DNA pellet was kept for further analysis. The pellet was washed using 1 milliliter of molecular grade 70% ethanol. The DNA and ethanol was vortexed to dislodge the pellet and spun for 5 minutes at 14,000 rpm and 4°C. After discarding the liquid, the transparent pellet is then dried using a speed vacuum, and resuspended in 100 microliters of TE buffer. The overall gDNA concentration and purity for each sample were assessed using the Nanodrop spectrophotometer. To determine the approximate size of the DNA yield, a 1% agarose gel was ran as a visual detection.

PCR Amplification

Identification of cellulose-degrading bacteria was achieved by amplifying the V1-V3 region of the 16S ribosomal RNA (rRNA) nucleotide gene sequence. A barcoded primer set was used to amplify the fragment from different samples. A 25 microliters Polymerase Chain Reaction (PCR) was performed using a thermocycler. Each

reaction included DNA taq polymerase (0.25 microliters), 10X buffer (2.5 microliters), ddH2O (17.75 microliters), Mg^{2+} (1 microliter), dNTP (0.5 microliters), template DNA (1 microliter), the forward (1 microliter) and reverse (1 microliter) primers. There were 35 cycles during amplification, with each cycle including a denaturation step at 95°C for 30 seconds, an annealing step at 65°C for 30 seconds, and an extension step at 72°C for 1 minute. The PCR reaction ended with extension at 72°C for 10 minutes.

The purity and yield of PCR products were accessed using Nanodrop 2000 spectrophotometer and 1% agarose gel. Excess dNTPs and primers were removed from the PCR reaction through purification using *NucleoSpin Extraction Kits* (Clontech Laboratories) in preparation for sequencing. For purification, each 20 microliters of PCR reaction was adjusted to 100 microliters with TE buffer. Once adjusted, 400 microliters of buffer NT2 (included with NucleoSpin kit) was mixed with each sample. The 500 microliters of reaction/buffer mixture was pipetted into a spin column inserted into a 2-milliliter collection tube. Each column was centrifuged at room temperature for 1 minute at 11,000x g, discarding the flow-through in the collection tube. The spin column was then reinserted into a new 2ml collection tube. 600 microliters of buffer NT3 was added to the spin column and centrifuged at room temperature for 1 minute at 11,000x g, discarding the flow-through. To remove residual NT3 buffer from the membrane filter, 200 microliters NT3 was added to the spin column reinserted into the 2-milliliter collection tube. The column was then centrifuged at room temperature for 3 minutes at 11,000x g. The spin column containing the purified PCR product was inserted into a clean 1.5-milliliter microcentrifuge tube. 50 microliters of Buffer NE was added to the column and the purified product was incubated at room temperature for one minute. Once

incubated, each sample was centrifuged at room temperature for 1 minute at 11,000x g. The purity and yield were analyzed using the Nanodrop spectrophotometer and 1% agarose gel.

Sequencing

The sequencing was completed using 454 sequencing technology. Sequencing by synthesis (SBS) technology uses four fluorescently- labeled nucleotides to sequence the tens of millions of clusters on the flow cell surface in parallel. For Roche 454 sequencing approach, library fragments are mixed with a population of beads whose surfaces carry oligonucleotides complementary to the 454 specific adapter sequences on the fragment library, so each bead is associated with a single fragment (Mardis 2008). Each of these fragment-bead complexes is isolated into individual oil-water micelles that also contain PCR reactants, and thermal cycling of the micelles produces approximately one million copies of each DNA fragment on the surface of each bead (Mardis 2008). These amplified single molecules are then sequenced in mass. First the beads are arrayed into a picotiter plate that holds a single bead in each of several hundred thousand single wells, which provides a fixed location at which each sequencing reaction can be monitored (Mardis 2008). Enzyme containing beads that catalyze the downstream pyrosequencing reaction steps are then added to the PTP and the mixture is centrifuged to surround the agarose beads. On the sequencer, the PTP acts as a flow cell into which each pure nucleotide solution is introduced in a step- wise fashion, with an imaging step after each nucleotide incorporation step. The PTP is seated opposite a CCD camera that records the light emitted at each bead. (Mardis 2008) The first four nucleotides on the adapter fragment adjacent to the sequencing primer added in library construction correspond to

the sequential flow of nucleotides into the flow cell. This strategy allows the 454 base-calling software to calibrate the light emitted by a single nucleotide incorporation (Mardis 2008). However, the calibrated base calling cannot properly interpret long stretches (>6) of the same nucleotide so these areas are prone to base insertion and deletion errors during base calling. By contrast, because each incorporation step is nucleotide specific, substitution errors are rarely encountered in Roche/454 sequence reads. The raw reads are processed by the 454-analysis software and then screened by various quality filters to remove poor-quality sequences, mixed sequences, and sequences without the initiating sequence (Mardis 2008).

Metagenomic Analysis

Bacterial isolates were categorized using Ribosomal Database Project (RDP) Analysis from 16S rRNA sequencing data. Once files are converted from FASTQ to FASTA, the RDPTools multi-classifier program allows accurate taxonomic assignments ranging from domain to genus. This analysis allows us to characterize a population of cellulolytic bacteria ranging from the kingdom to species taxonomic ranks. The RDP Classifier rapidly and accurately assigns sequences into taxa with bootstrap value, an estimate of confidence for each assignment**.**

Graphical representations of sample bacteria compositions were achieved through R Statistical Computing Analysis Program. Classified and hierarchy files were first produced using RDPtools. Once generated, further processing of classifier output was carried out using the Phyloseq package in R. Sample file including sample titles, descriptions, and variables was created in Excel and saved as a .csv file.

Visualization of the microbiome composition at the various taxonomic levels was achieved for each sample using KronaTools, which creates a hierarchical data presentation in a .html file, which in turn could be viewed in Firefox web browser. For this analysis, the sequence data file for each sample was first classified using the RDPTools, which created the file that was used by KronaTools as the input file.

Biochemical Analysis

Cellulose Quantification

Cellulose concentration was measured according to "estimation of cellulose 1.9" method. 3 milliliters of Acetic/nitric reagent consisting of 150 milliliters of 80% acetic acid and 15 milliliters of concentrated nitric acid was added to 1ml of sample into a test tube. Once mixed, the 4 milliliters reagent and sample mixture was heated in a water bath at 100°C for 30 minutes. Each reaction mixture was cooled and centrifuged at max speed for 20 minutes. The supernatant was removed and the remaining cellulose washed with distilled water. 10 milliliters of 67% sulphuric acid was added to the cellulose and allowed to incubate at room temperature for 1 hour. Each sample was then diluted to 100 milliliters and to 1 milliliter of the diluted solution, 10 milliliters of anthrone reagent consisting of 200 milligrams anthrone reagent and 100 milliliters sulphuric acid was added to the test tube and mixed well. The reaction tubes were then heated in a boiling bath for 10 minutes. Once cooled, the absorbance was read at 600 nanometers using Nanodrop spectrometer. Cellulose concentration was calculated through a standard curve that was created using a series of dilutions as standards ranging from 5 milligrams/milliliter to 100 milligrams/milliliter.

Glucose Quantification

Supernatant removed from cellulose culture was used to measure the relative amounts of glucose used by cells and remaining in solution. Each sample was spun at 14,000 x g for 45 minutes to separate cells from glucose in the sample. Once cellular pellet was precipitated, the supernatant containing glucose was transferred out and used for the analysis. 3 milliliter of DNS reagent was added to the 3 milliliters of glucose-containing supernatant from the previous step. Each reaction tube was heated in a water bath for 15 minutes. 1 milliliter of 40% potassium sodium tartrate (Rochelle salt) was added to the 6 milliliters of mixture to stabilize color. The absorbance was read using Nanodrop spectrometer at 510 nanometers. A standard curve was created from a series of glucose solutions ranging from 500 milligrams/3 milliliters to 50 milligrams/3 milliliters, which was treated in the same method as described above for the sample cultures. The bestfit linear line from the standard curve was then used to calculate the glucose concentrations from each sample, which was then used to calculate the total amount of glucose remaining in each sample after the cellulose culture.

CHAPTER III

RESULTS

Bacterial Diversity of Termite Samples Without Selective Culture

Characterization of the relative composition of the bacterial community in Colony 1 of the Termite sample (C1) collected from the East Woods of VSU campus shows great bacterial diversity. The bacteria found in this colony of termites are highly diverse with Firmicutes, Bacteroidetes, and Proteobacteria being the three major groups at the phylum level. The Firmicutes is dominated by Clostridia, with Bacilli being the second major group. However, the Bacteroidetes and Proteobacteria each is dominated exclusively by one class, namely, Bacteroidia and deltaproteobacteria, respectively. The relative abundance of cellulose-degrading bacteria by phyla, are described in detail in Figure 2. The relative abundance of cellulose-degrading bacteria by class, are described in detail in Figures 3-5.

Bacterial Diversity from Cellulose-Selective Culture

Analysis of the 16S rRNA gene of the "selective" (cellulose) culture indicates an abundance of three major phyla amongst samples: Proteobacteria, Firmicutes, and Bacteroidetes. However, the abundance of each phylum varies among samples. The relative abundance of Cellulose-degrading bacteria by phyla, are described in detail in Figure 7. There was great similarity amongst the decaying leaf #1, twig #1, and twig #2 samples from the Bear Creek State Park. Each of these samples was comprised mainly of

the phylum Proteobacteria, with varying but generally very small percentages of Firmicutes (Figure 2). The Proteobacteria in the soil samples were further dominated by gammaproteobacteria, which in turn is dominated by Enterobacteriaceae. At the genus level, however, the three samples show different compositions. The two twig samples are nearly identical to each other with Escherichia/Shigella being the most dominant group (making 47% and 45%, respectively), followed by Serratia (28% in both samples). Other major groups in the two twig samples include Enterobacter (7% and 9% respectively), Salmonella (7% and 8% respectively), Citrobacter (3% and 5%), and Buttiauxella (2% in both). However, the decaying leaf sample contains very little (only 2%) of Escherichia/Shigella but instead is dominated by Serratia (89%), though it also contains small amounts of Enterobacter (3%) and Buttiauxella (2%).

Six out of the ten other samples, including the two termite samples, the soil sample, and the three samples from sheep rumen #2, show predominance by one main phylum, Proteobacteria (Figure 7). Amongst the three different filtrations of the sheep rumen #2, there is a dominance of Proteobacteria with very similar numbers of sequence reads. In contrast, the sheep rumen #1 contains very little Proteobacteria and is dominated by the phylum Firmicutes or Bacteroidetes (Figure 7). The composition of sheep rumen #1 also varies amongst the three filtrations. Our analysis shows a high abundance of Firmicutes with a considerable amount of Bacteroidetes in the sheep rumen #2 unfiltered and combined wash-off samples. However, the 0.22 micrometer filtered sample of rumen #2 shows an abundance of Bacteroidetes, which tend to be smaller in size compared to the Firmicutes found in the other rumen #2 filtrations.

Metagenomic analysis of the termite gut for both termite samples shows a high level of Proteobacteria (Figure 7). Proteobacteria makes the single dominant group in the gut of the termites collected by the Appomattox River south to the Virginia State University campus. However, besides Proteobacteria, Firmicutes is another major group of bacteria found in the termite guts of the C1 colony collected on the campus of VSU (Figure 7). Similarly, the soil sample collected at the same site as the C1 termite colony also contains Proteobacteria and Fermicutes as the two major groups of bacteria and even with the similar relative frequency between the two phyla. Trace amounts of Bacteroidetes also appeared in the termite and soils samples.

The decaying plant leaf #2 community is largely dominated by Firmicutes with a slight abundance of Acidobacteria. Interestingly, Acidobacteria were found in the soil and sheep rumen collections. This association is not surprising considering the relationship of soil, plant growth, and plant consumption and digestion by ruminants.

To further characterize the cellulose-degrading bacteria at detailed level, each of the three major phyla found in the selective cultures were analyzed at the class level. For proteobacteria, the two termite samples contain noticeably different microbiomes at the class level while they are very similar at the phylum level. The termites from the Appomattox River show a great abundance of betaproteobacteria whereas the termites from the East Woods on VSU campus show very low abundance of betaproteobacteria with only less than 500 sequence reads. In contrast, there was a clear absence of gammaproteobacteria in the Appomattox River termite colony but great abundance in the VSU East Woods colony. Similarly, the soil sample collected at the same site as the VSU East Woods termite colony is also dominated by gammaproteobacteria.

For sheep rumen #2, gamma-proteobacteria is the dominant group of proteobacteria in the unfiltered sample. However, in the combined filtered sample, both gammaproteobacteria and alphaproteobacteria are similarly abundant as the co-dominant proteobacterial groups. In contrast, the 0.22 micrometer filtration of rumen #2 is dominant by alphaproteobacteria. For sheep rumen #1, the unfiltered sample is dominated by the betabacteria, which is different from sheep rumen #2. The combined filtered sample of rumen #1, however, is dominated by gammaproteobacteria, while the 0.22 micrometer filtered rumen #1 sample contains both gammaproteobacteria and alphaproteobacteria in similar abundance. It should be noted though, the abundance of each of these groups of proteobacteria in rumen #1 samples is much lower than that in rumen #2 (Figures 12, 14, & 19), consistent with the overall low abundance of proteobacteria in rumen #2 samples (Figure 7).

While the dominant class of Proteobacteria varies among samples, the same class of Firmicutes, Bacilli, seems to dominate most of the samples, followed by Clostridia. As shown in Figure 2, Bacilli is the most dominant group of Firmicutes in both termite samples, the soil sample, the decaying leaf sample, and the unfiltered and combined filtered rumen #1 samples, while Clostridia is the dominant group of the rumen #1 0.22 micrometer filtered sample and all the three rumen #2 samples.

Rumen #1 0.22 micrometer filtration and rumen #1 combined wash-off were the only samples that contain any significant amount of bacteroidetes. Flavobacteriia and Sphingobacteriia are the two major classes of bacteroidetes in the two samples. While Flavobacteriia dominates the rumen #1 0.22 micrometer sample with Sphingobacteriia as the second largest class, the latter is the single dominant class of bacteroidetes in rumen

#1 combined wash-off sample. The relative abundance of Cellulose-degrading bacteria by class, are described in detail in Figures 8-10.

Comparison of Bacterial Diversity With or Without Cellulose Selective Culture

Direct comparison in microbial diversity with and without cellulose selective culture can be made for the termite colony collected at the same site at the East Woods on the VSU campus. For the termite sample from the East Woods, the share of Proteobacteria in the bacterial community after the cellulose culture has increased while that for Firmicutes has decreased and the abundance of Bacteroidetes was significantly reduced after the cellulose culture. The change for Proteobacteria and Firmicutes was more dramatic at the class level. For Proteobacteria, the dominant class before cellulose selective culture is deltaproteobacteria while it is gammaproteobacteria. For Firmicutes, the dominant class has changed from Clostridia before cellulose culture to Bacilli after the culture. These changes in relative frequency suggest the difference among these groups of bacteria in utilizing cellulose. The microbiome of each sample are described in detail to the genus level in Figures 38-51.

Biochemical Analysis of Cellulolytic Activities of Selective Cultures

A biochemical analysis was performed on all samples cultured in the selective cellulose media. The concentration of cells was calculated using a standard curve plotting concentrations against the absorbance measured at optical density 600 nanometers. Measuring cell concentration, analysis shows the highest concentration of cells among the 0.22 micrometer filtrate from sheep rumen #2 and the combined wash-off from sheep rumen #1. Cell measurements at optical density 600 nanometers, are described in detail in

table 1. Cellulose was measured using a standard curve from concentrations and absorbance's read at optical density 630 nanometers. Each of the samples used at least 50% of the cellulose from the selective media during the culturing period. Cellulose content of standards and sample collections measured at optical density 630, are described in detail in tables 3 and 4. While quantifying the glucose content, results show the majority of the glucose in the samples had been used up during the cell growth process. Quantification of glucose was achieved through the use of standard curves plotting concentrations against absorbance's read at optical density 510 nanometers. Glucose content of standards and sample collections measured at optical density 510, are described in detail in tables 5 and 6.

The values outlined in Figures 6 and 11 (alpha diversity plot) directly correlates to the rarity of bacterial classes based on the total number of observed species, species captured once, and species captured twice (Hughes *et al.* 2001). Based on this diversity analysis, sheep rumen #1 contains the most rare classes among the selective cultures. The C2 colony of the termite hindgut collected along the Appomattox River shows the least amount of rare classes between these samples. Comparing the Chao1 index of the three filtrations of sheep rumen #1 samples reveal the most diversity among the combined wash-off of each filtration (C3) and a lower number of rare classes in the smallest filtration of the sample, 0.22 micrometer. Analysis of the sheep rumen #2 identifies the smallest of the three filtrations as having the most rare classes. Unlike the finding of sheep rumen #1, the unfiltered collection of sheep rumen #2 contains the least number of rare classes. Both samples of the termite hindgut displays nearly identical amounts of classes based on Chao1 diversity. The Simpson index measure the probability that two

bacteria taken at random from the sample would represent the same classification. Converting these values into percentages, it tells us all of the bacteria found within the selective cultures have at least an 80% chance of belonging to the same taxonomic group except for the termite hindgut from the Appomattox River and the sheep rumen #2 unfiltered. The relatively high Simpson Index indicates a lack of richness within samples and correlates to the core set of bacteria identified.

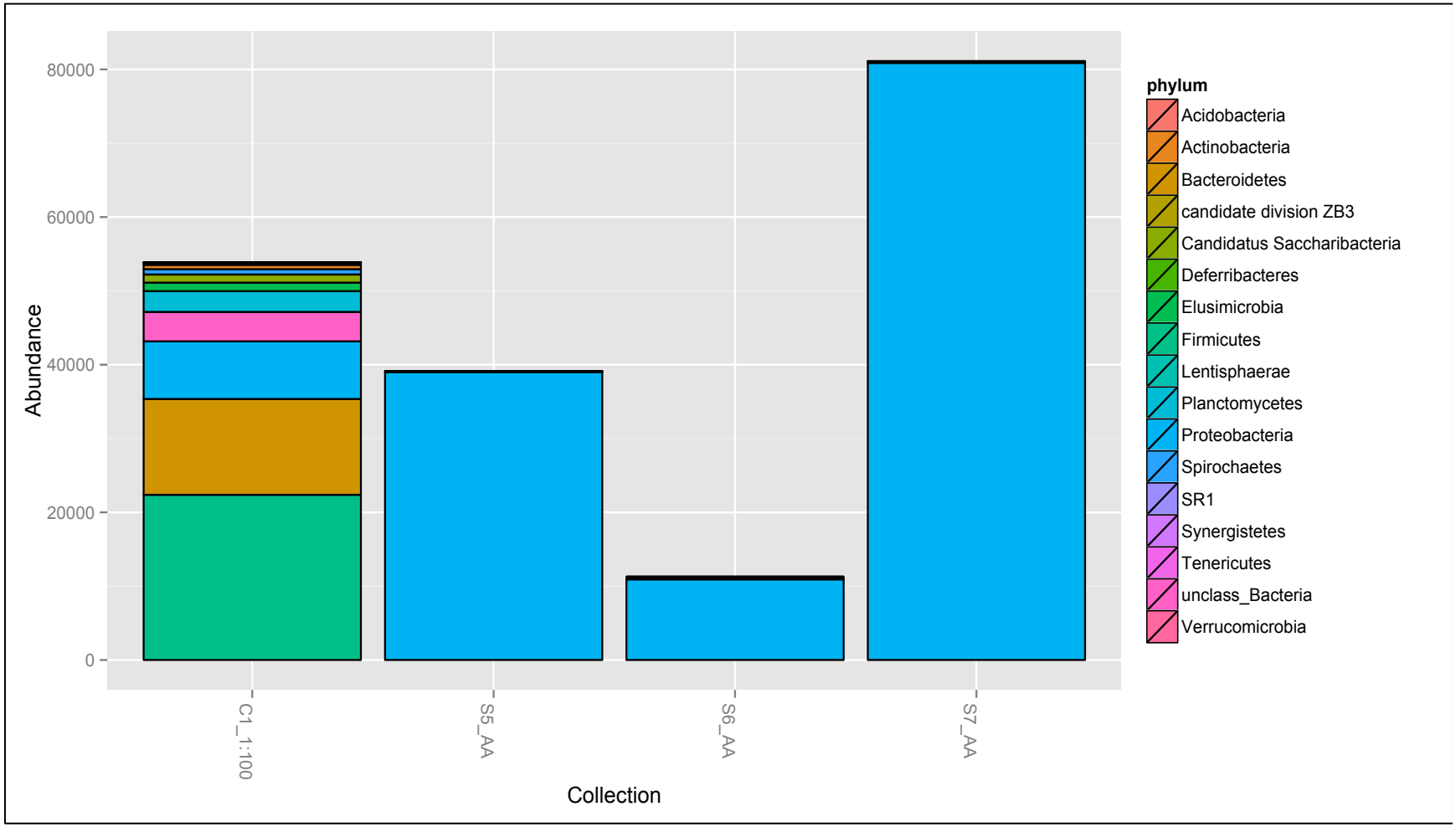

Figure 2 Relative abundance of bacteria at the phylum level from uncultured termite hindgut #1 sample and the selective culture of leaf sample #1 and the two twig samples

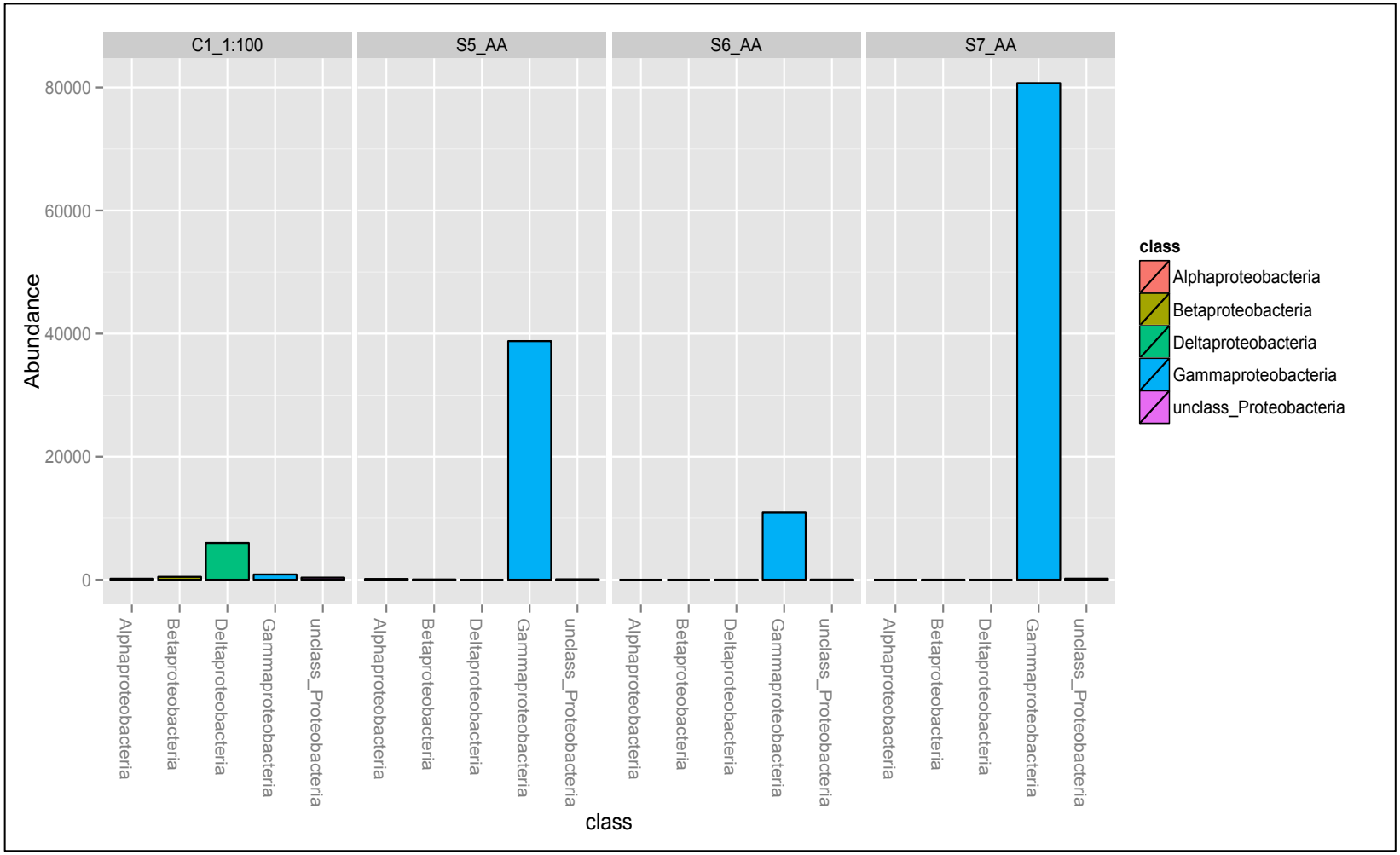

Figure 3 Relative Abundance of Proteobacteria at the class level from uncultured termite hindgut #1 sample and the selective culture of leaf sample #1 and the two twig samples

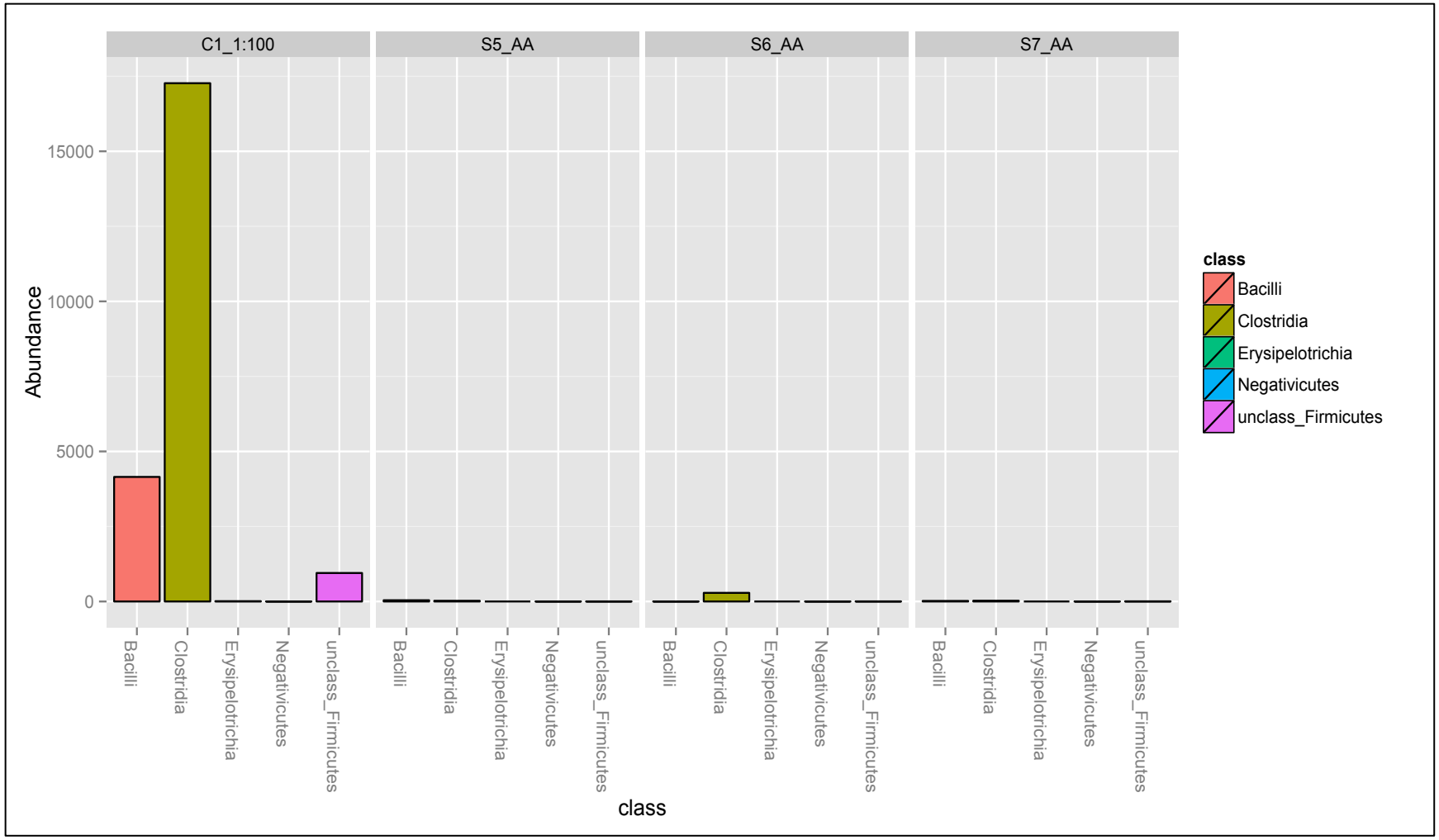

Figure 4 Relative abundance of Firmicutes at the class level from uncultured termite hindgut #1 sample and the selective culture of leaf sample #1 and the two twig samples

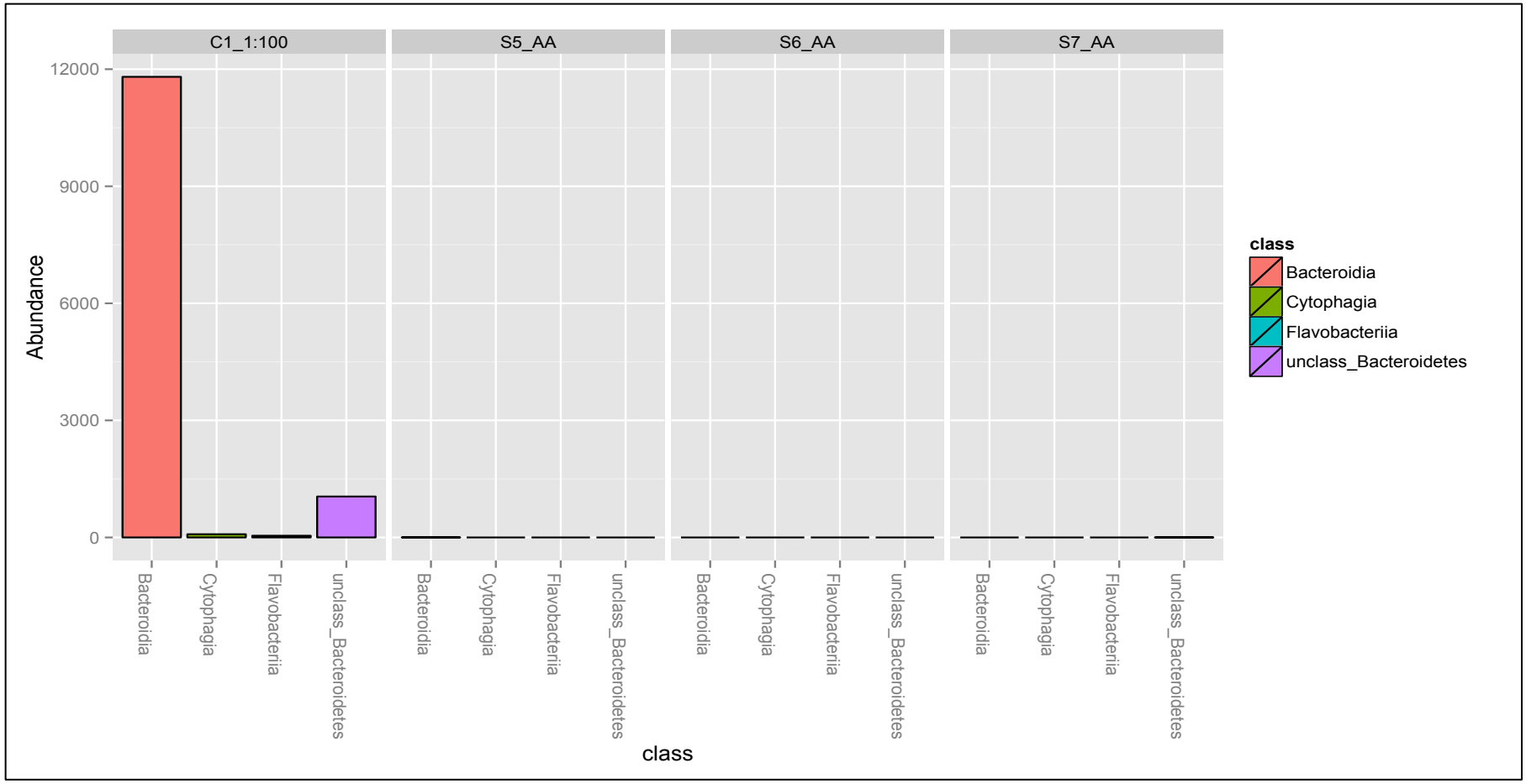

Figure 5 Relative abundance of Bacteroidetes at the class level from uncultured termite hindgut #1 sample and the selective culture of leaf sample #1 and the two twig samples

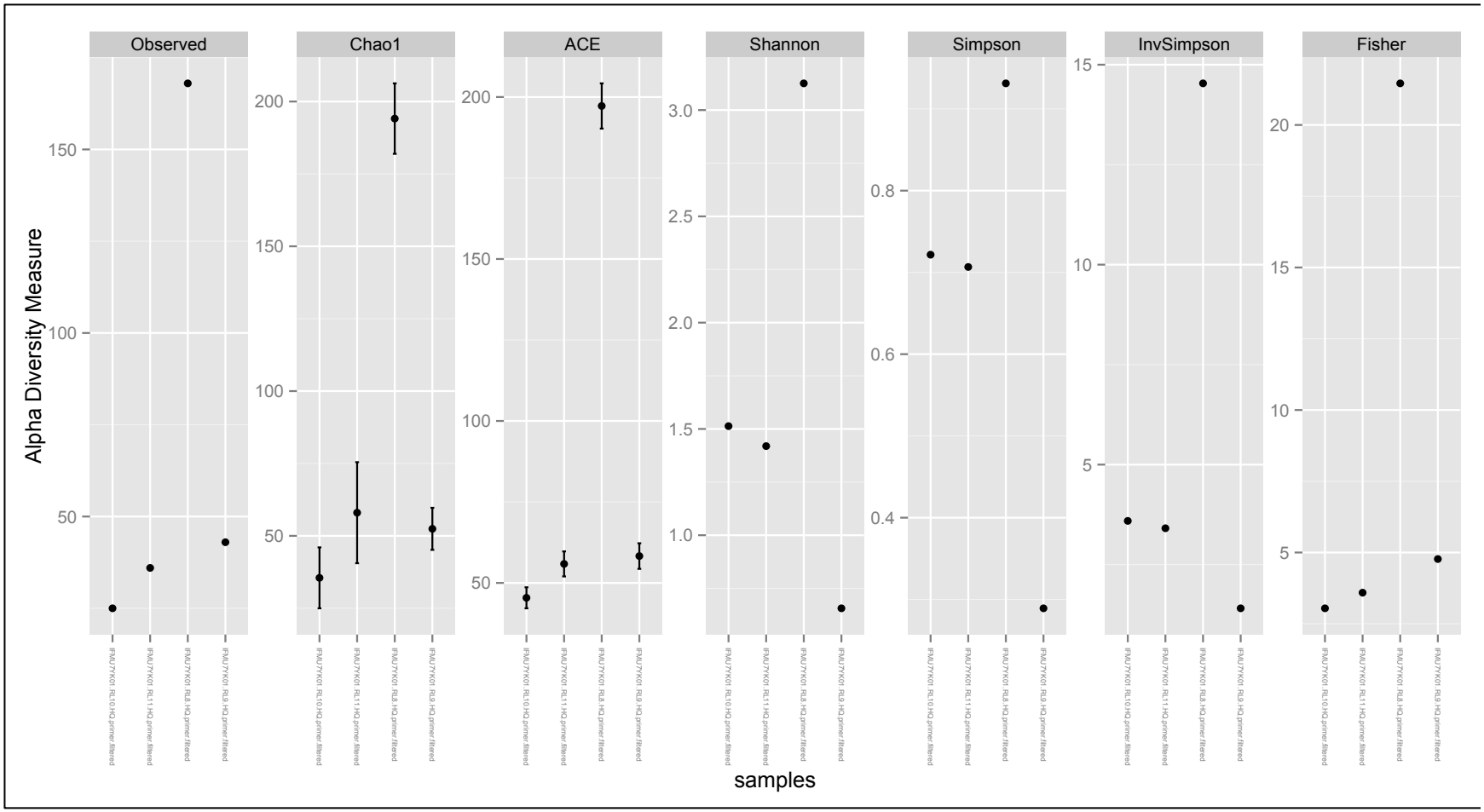

Figure 6 Alpha Diversity measures of the uncultured termite hindgut #1 and the selective culture of leaf sample #1 and the two twig samples

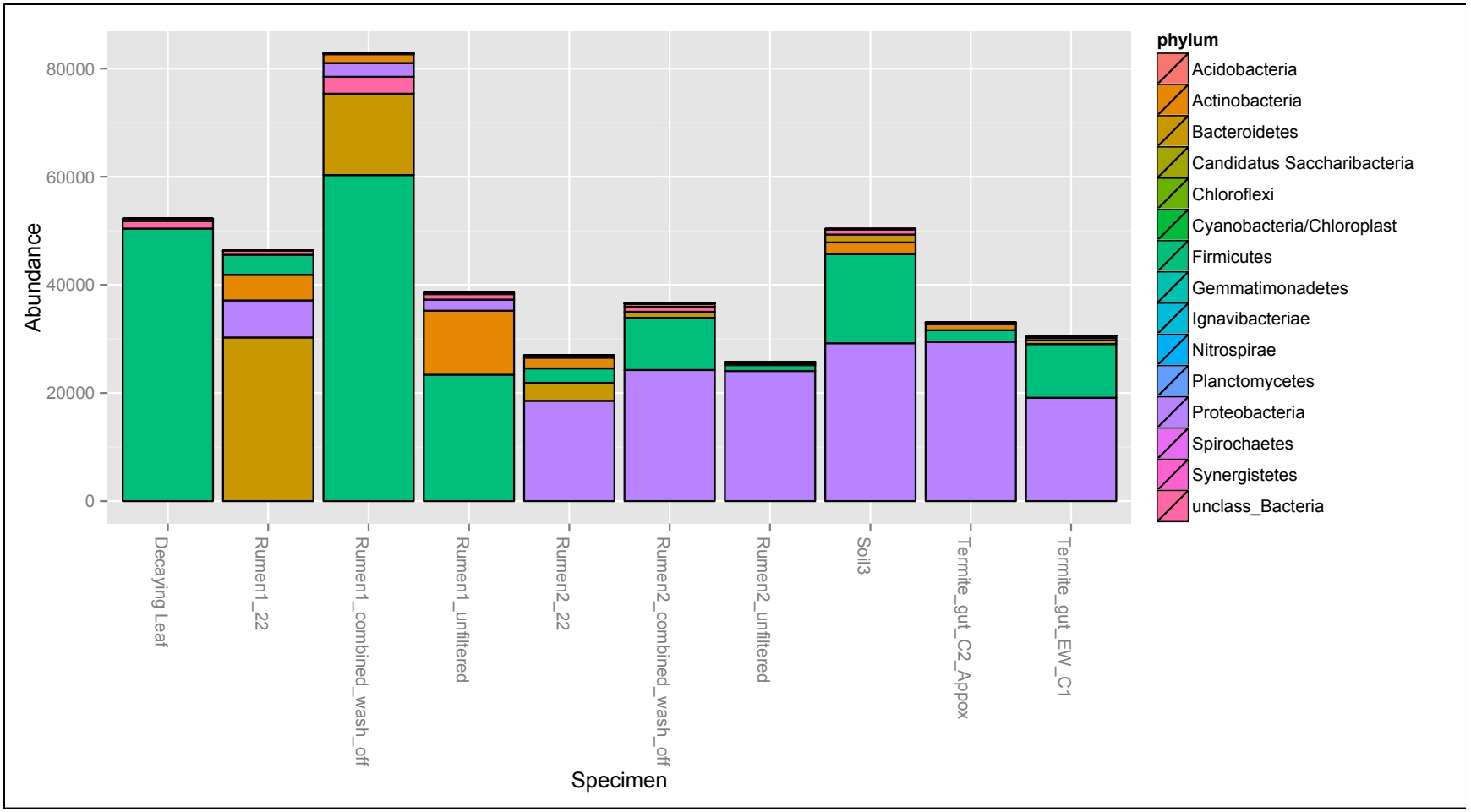

Figure 7 Relative abundance of Bacteria at the phylum level from the selective cultures of ten other samples

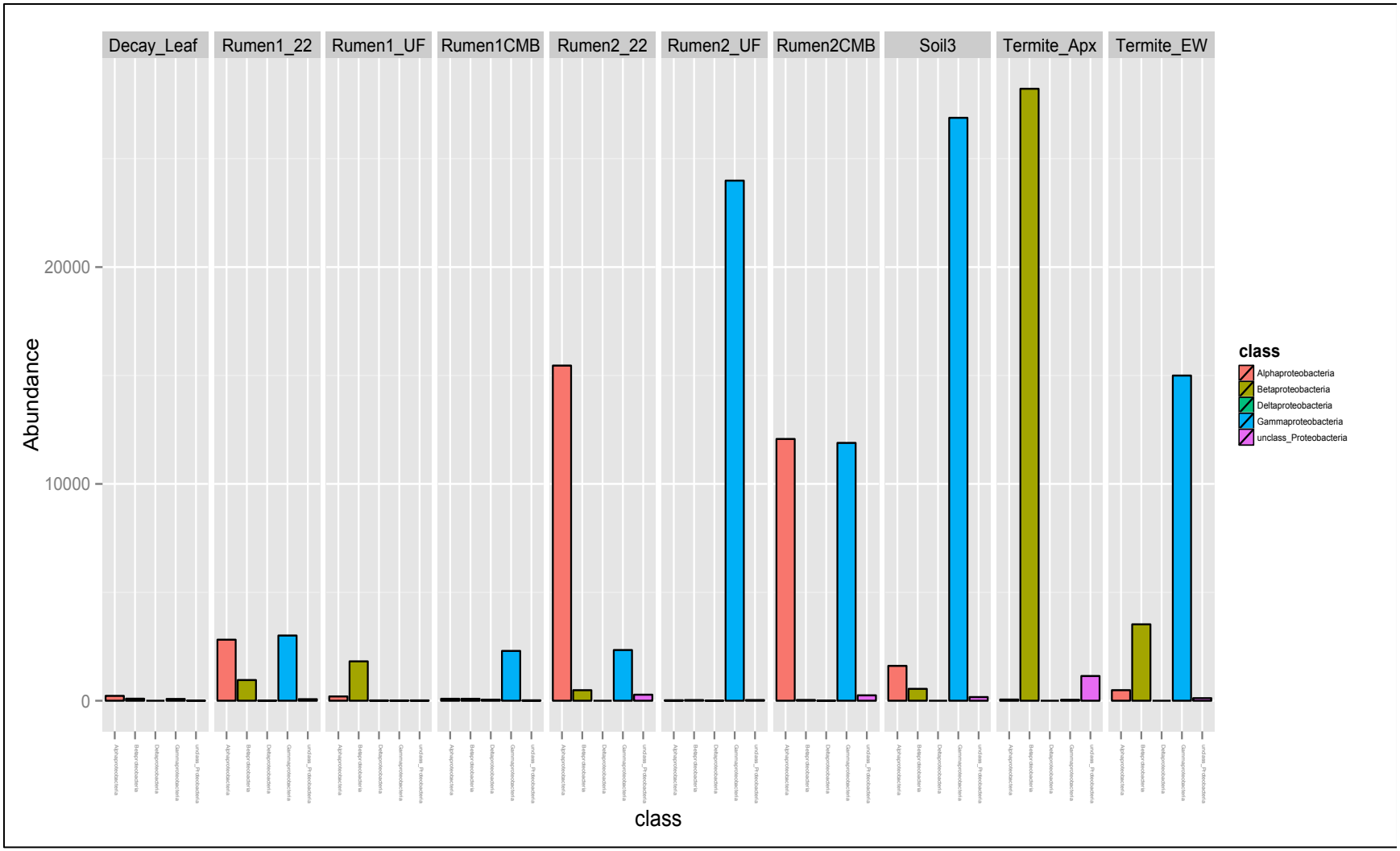

Figure 8 Relative abundance of Proteobacteria at the class level from the selective cultures of ten other samples

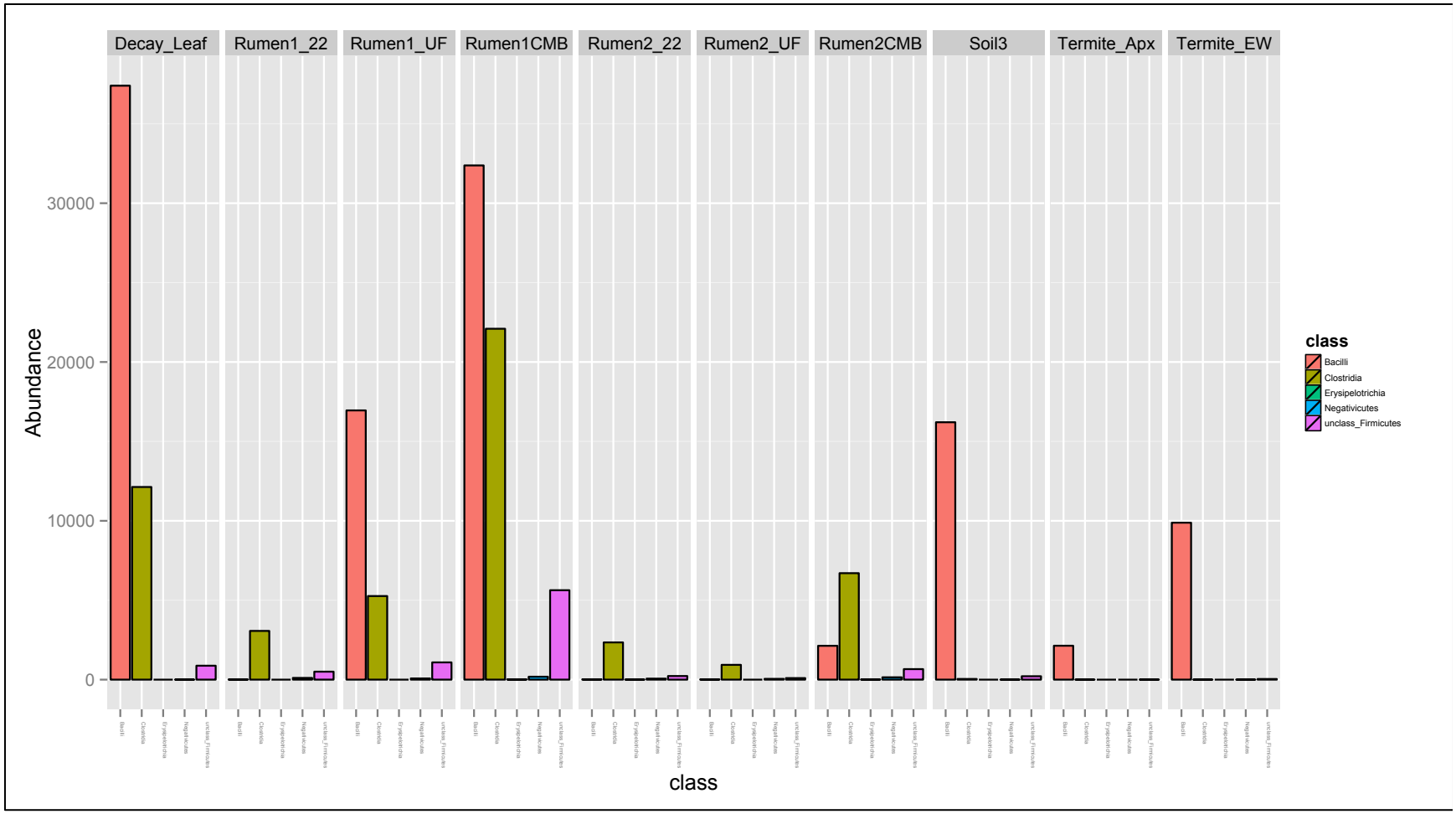

Figure 9 Relative abundance of Firmicutes at the class level from the selective cultures of ten other samples

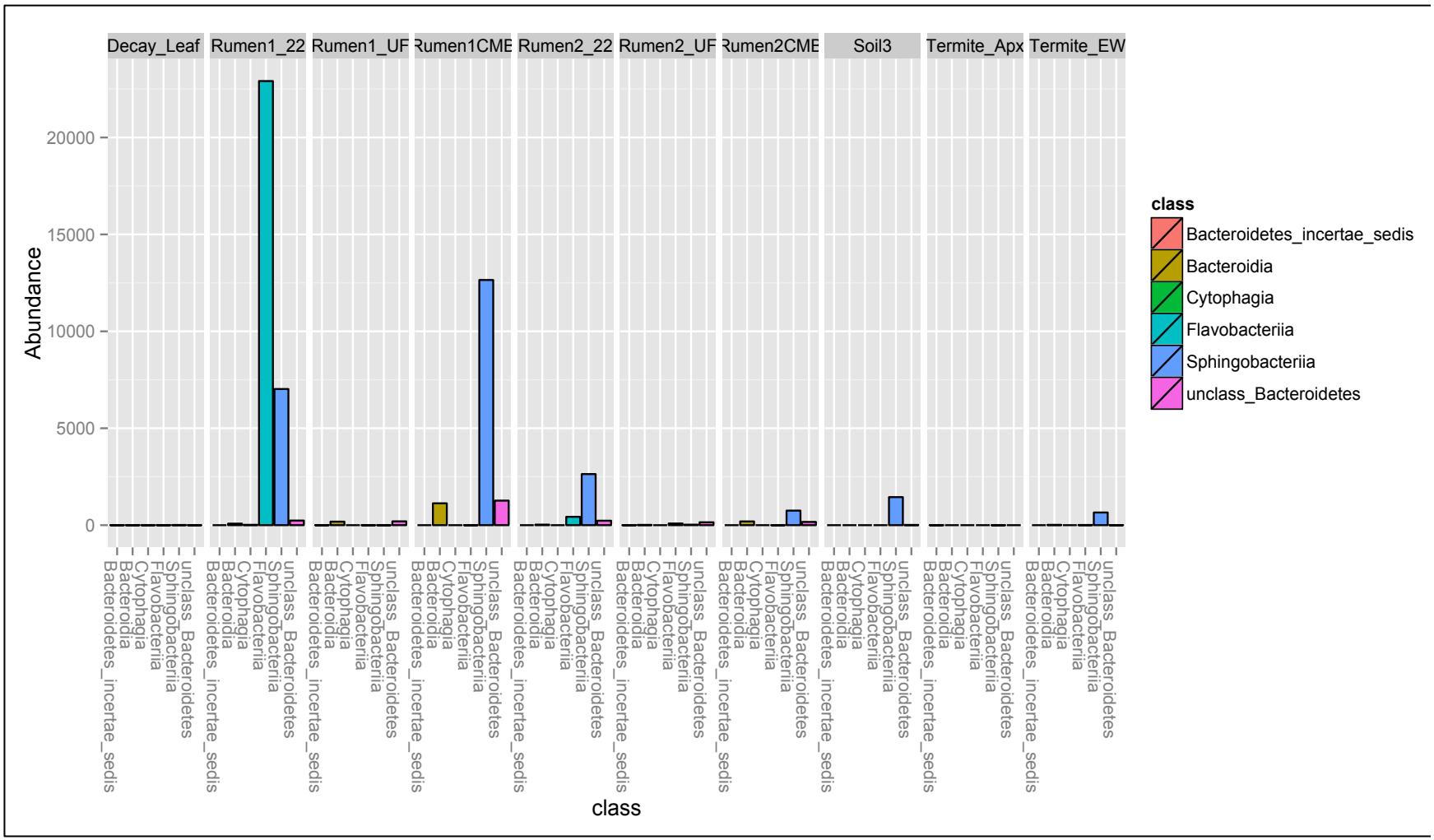

Figure 10 Relative abundance of Bacteroidetes at the class level from the selective cultures of ten other samples

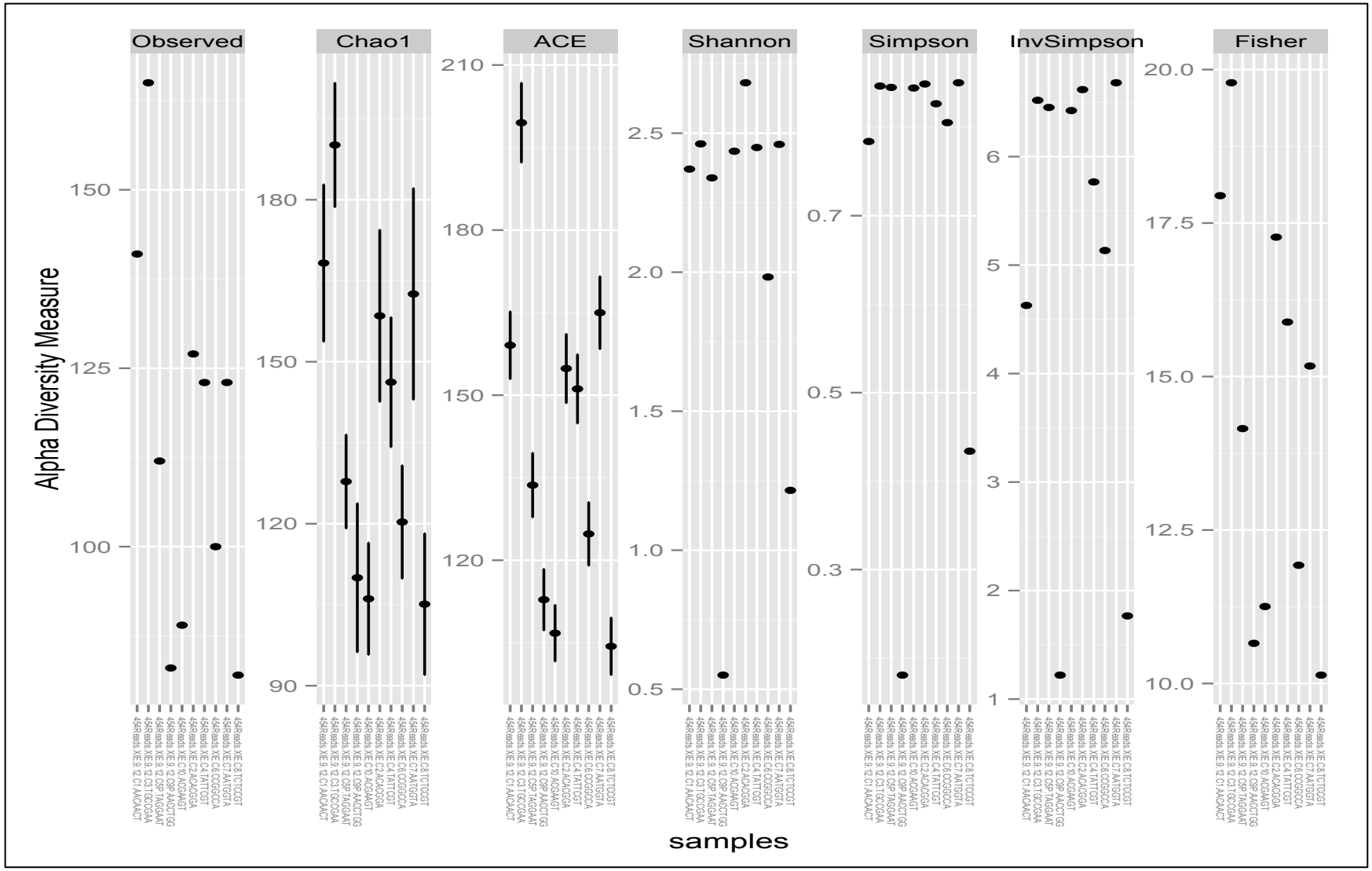

Figure 11 Alpha Diversity measures from the selective cultures of ten other samples

Figure 12 Microbiome of Termite hindgut without any culture from the phylum to the genus level

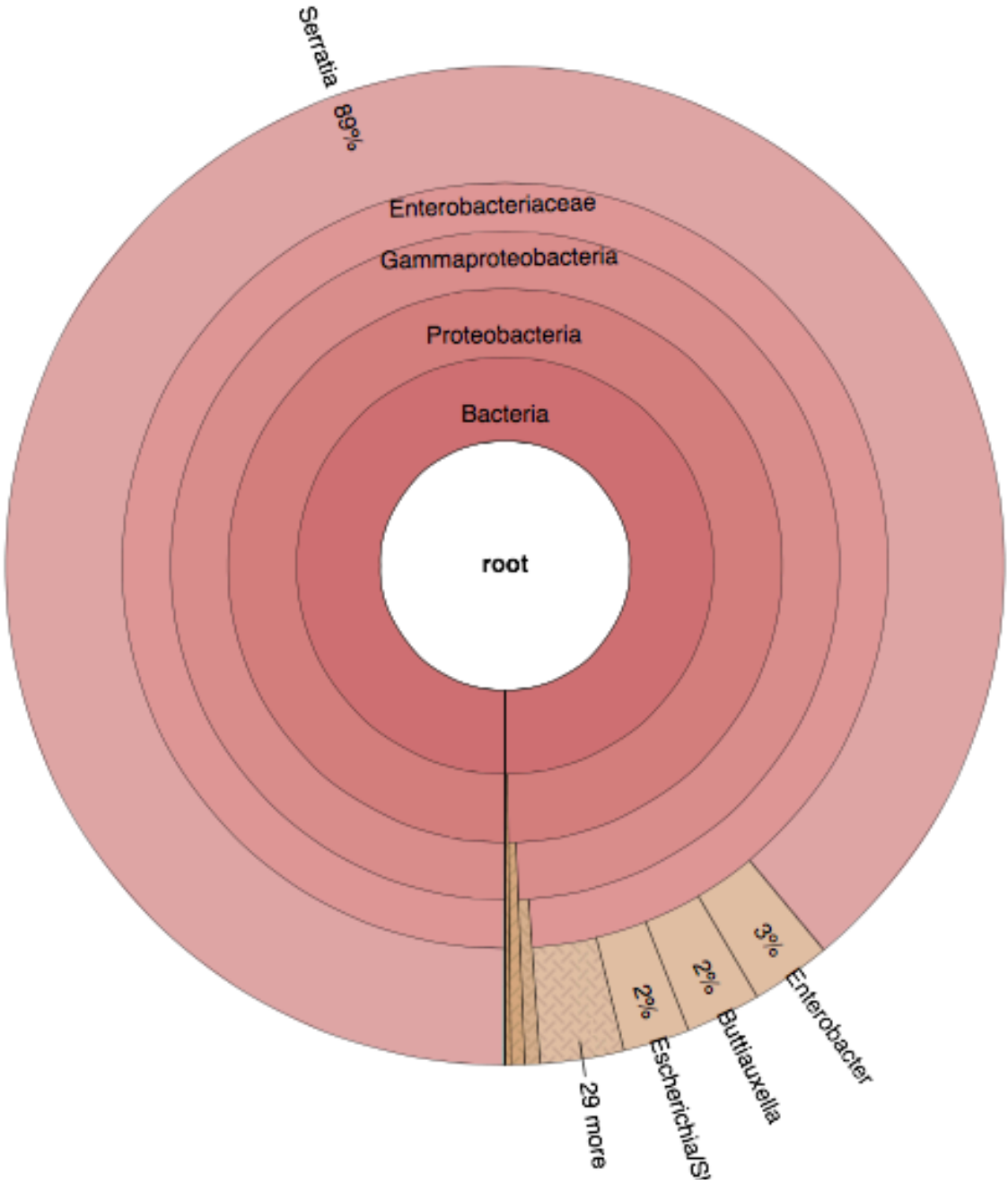

Figure 13 Microbiome of leaf sample #1 from the Bear Creek State Park cultured in selective cellulose media from the phylum to the genus level

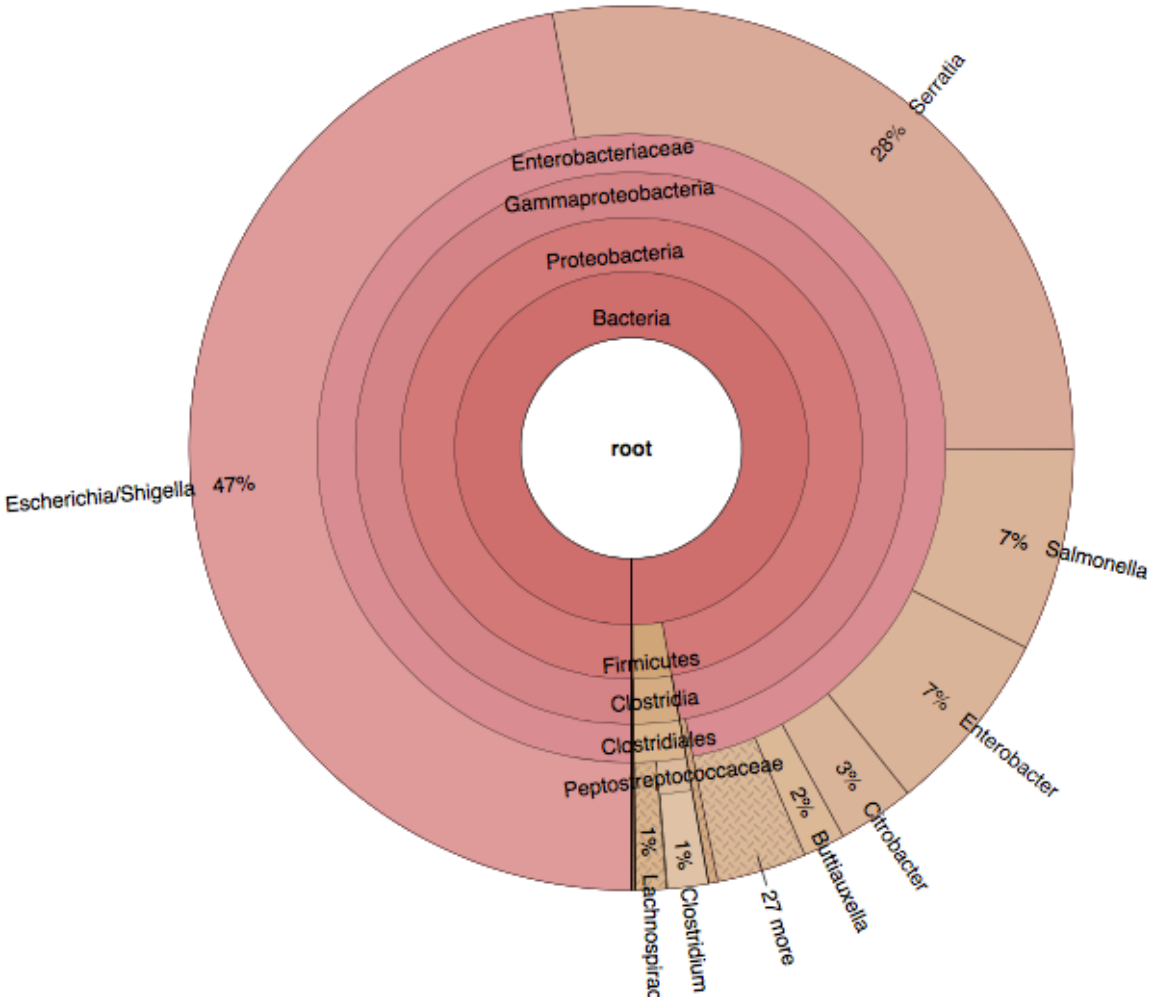

Figure 14 Microbiome of twig sample #1 form the Bear Creek State Park cultured in selective cellulose media from the phylum to the genus level

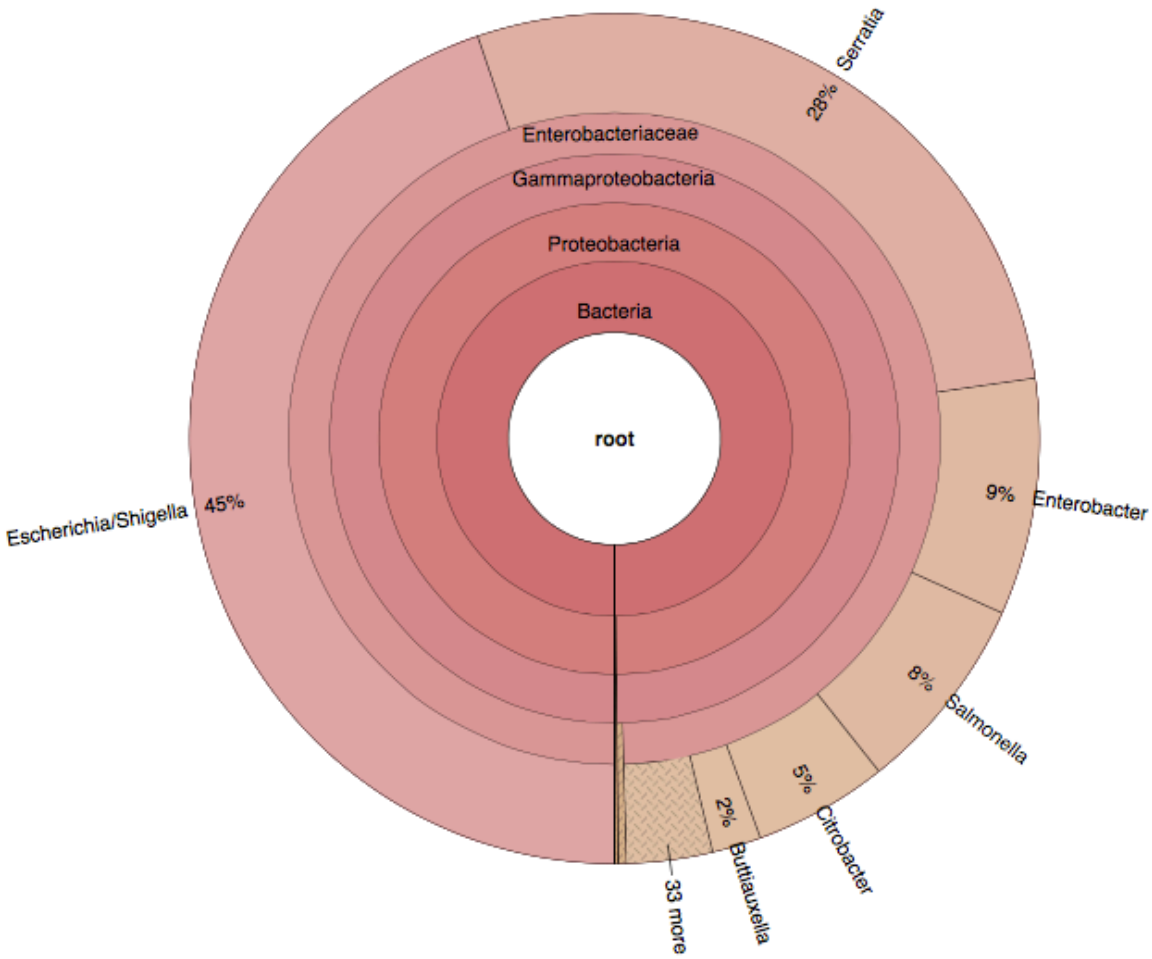

Figure 15 Microbiome of twig sample #2 from the Bear Creek State Park cultured in selective cellulose media from the phylum to the genus level

Figure 16 Microbiome of Sheep Rumen #1 0.22 micrometer filtrate cultured in selective cellulose media from the phylum to the genus level

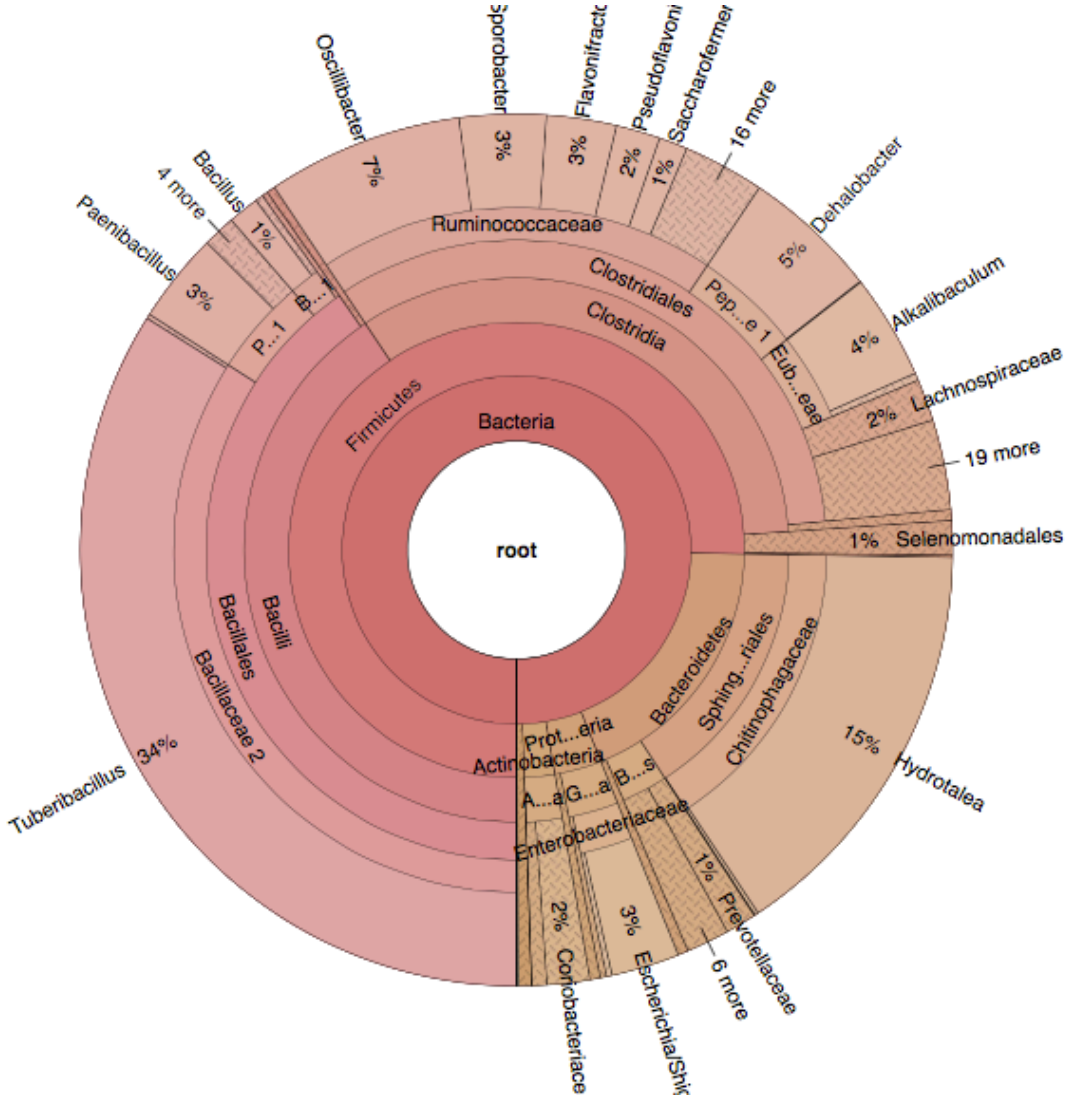

Figure 17 Microbiome of Sheep Rumen #1 combined wash-off cultured in selective cellulose media from the phylum to the genus level

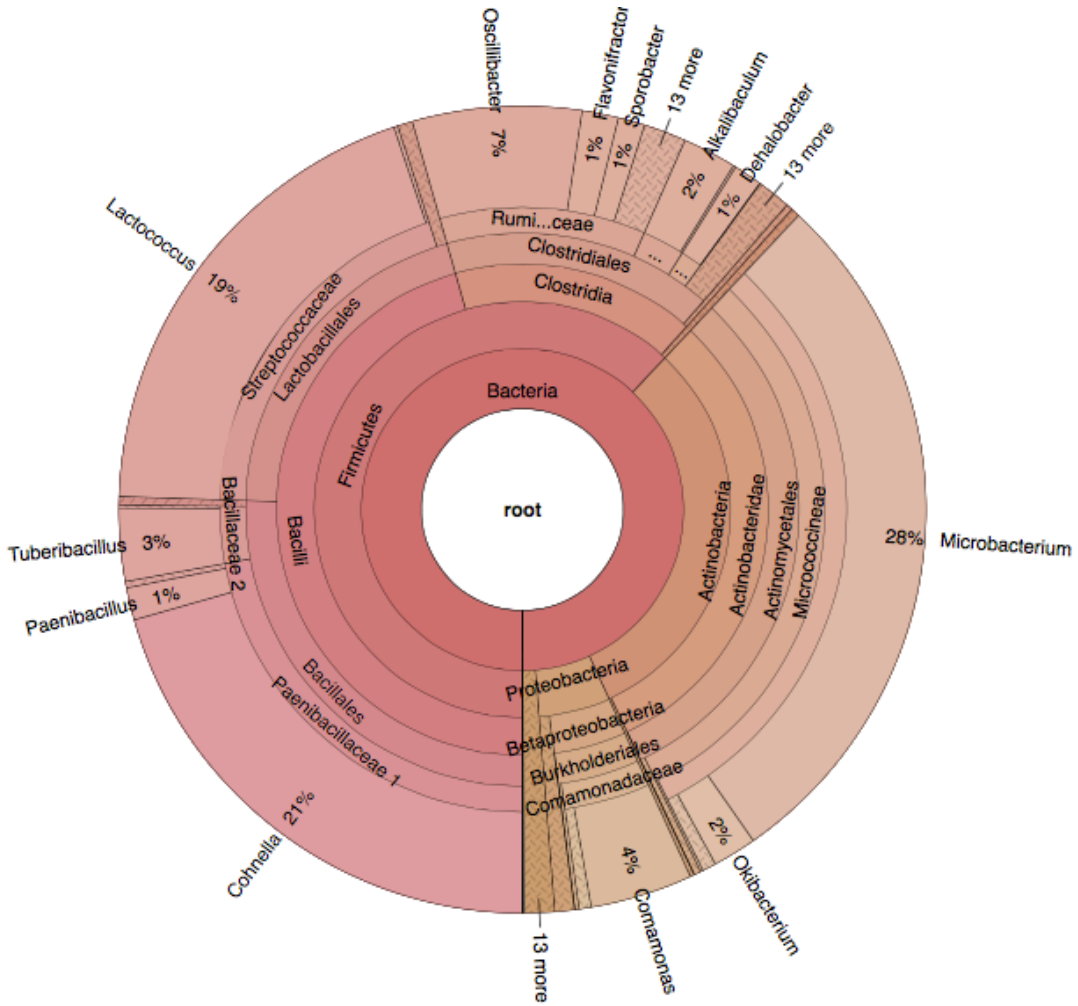

Figure 18 Microbiome of Sheep Rumen #1 unfiltered cultured in selective cellulose media from the phylum to the genus level

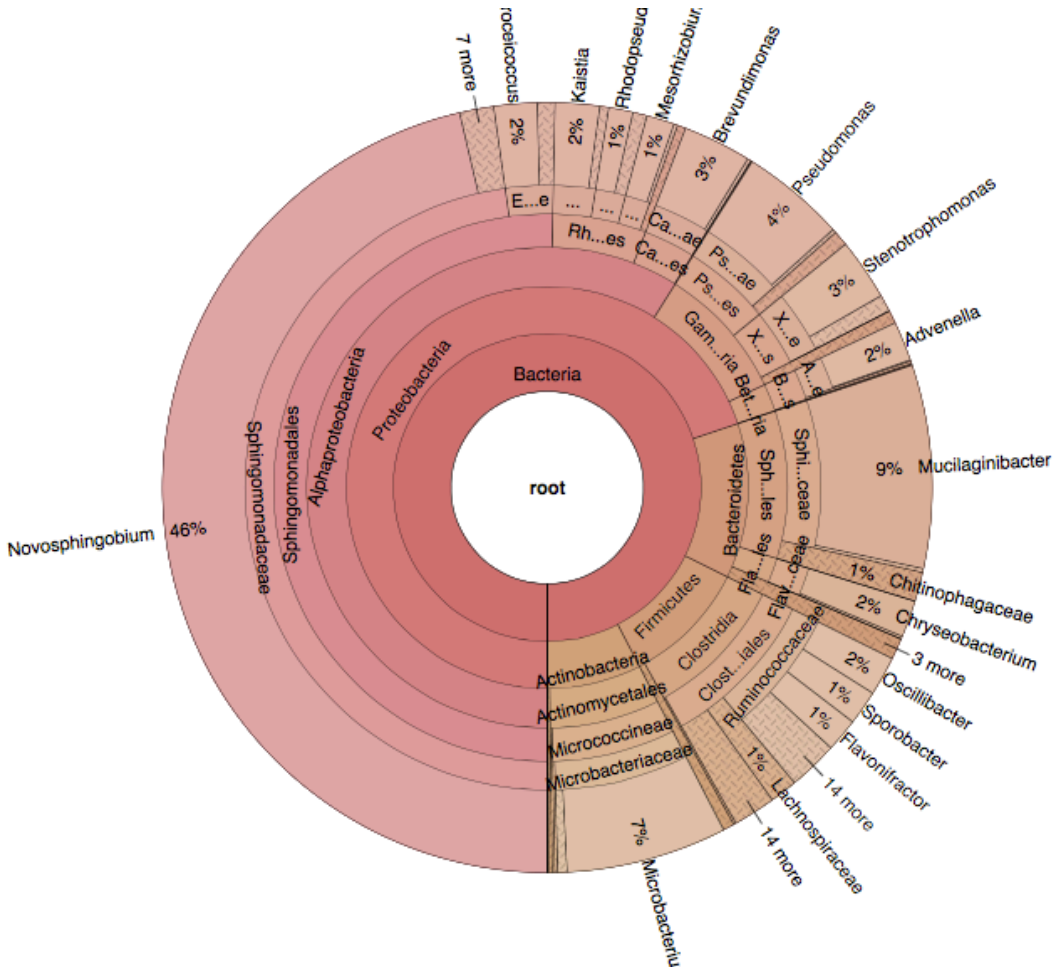

Figure 19 Microbiome of Sheep Rumen #2 0.22 micrometer filtrate cultured in selective cellulose media from the phylum to the genus level

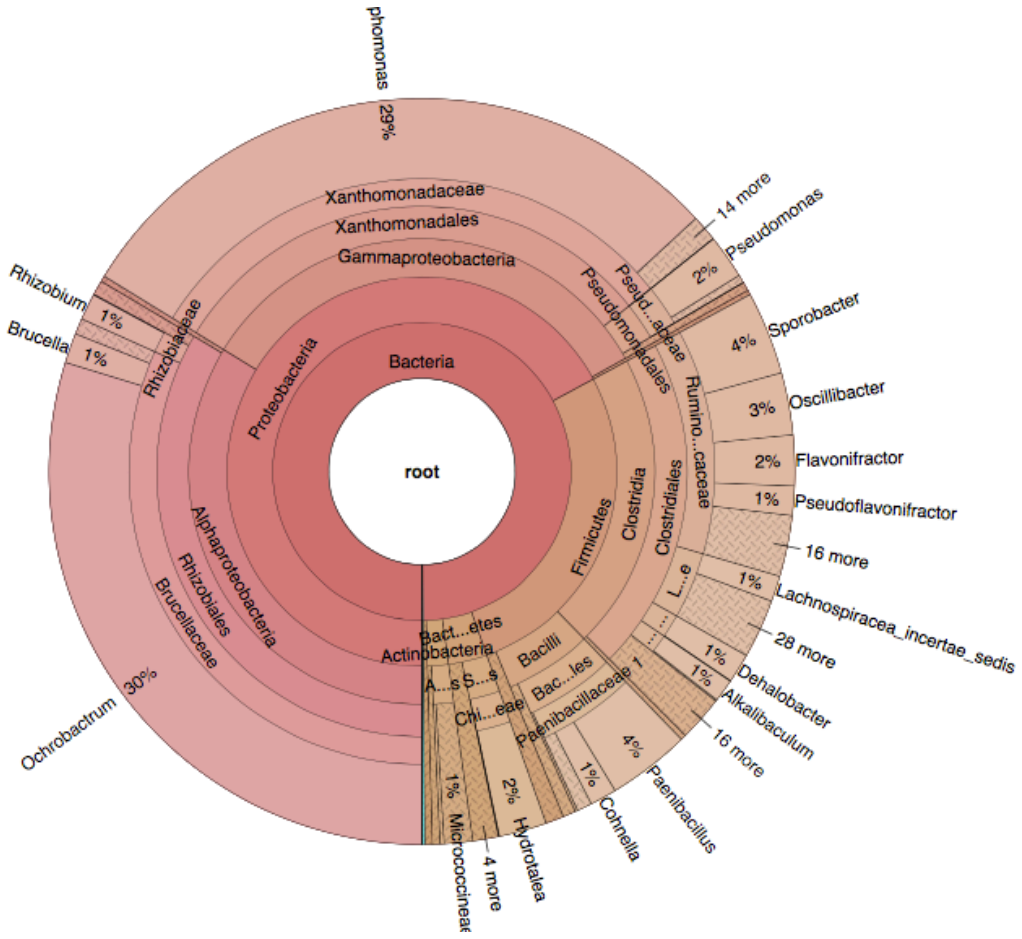

Figure 20 Microbiome of Sheep Rumen #2 combined wash-off cultured in selective cellulose media from the phylum to the genus level

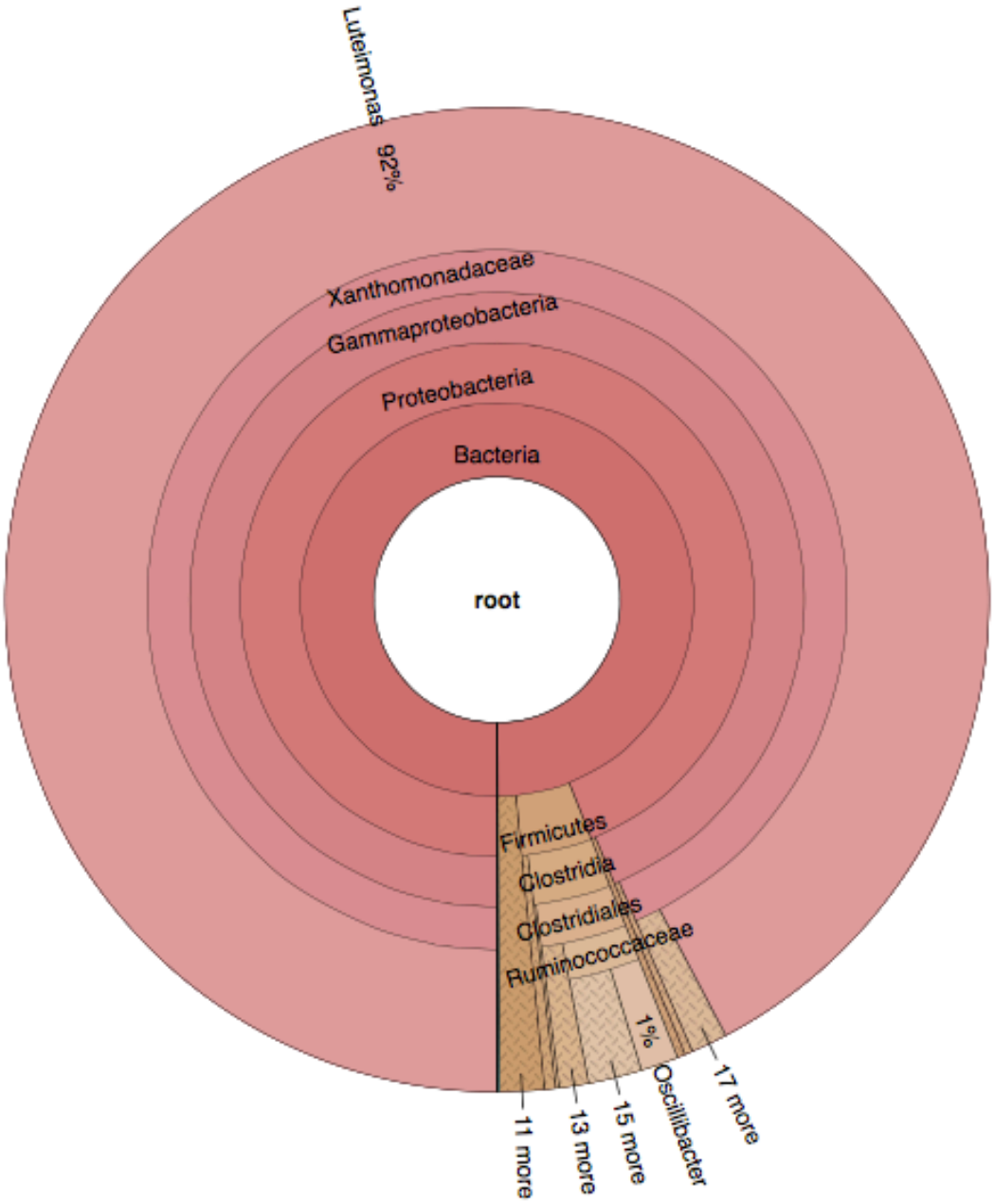

Figure 21 Microbiome of sheep rumen #2 unfiltered cultured in selective cellulose media from the phylum to the genus level

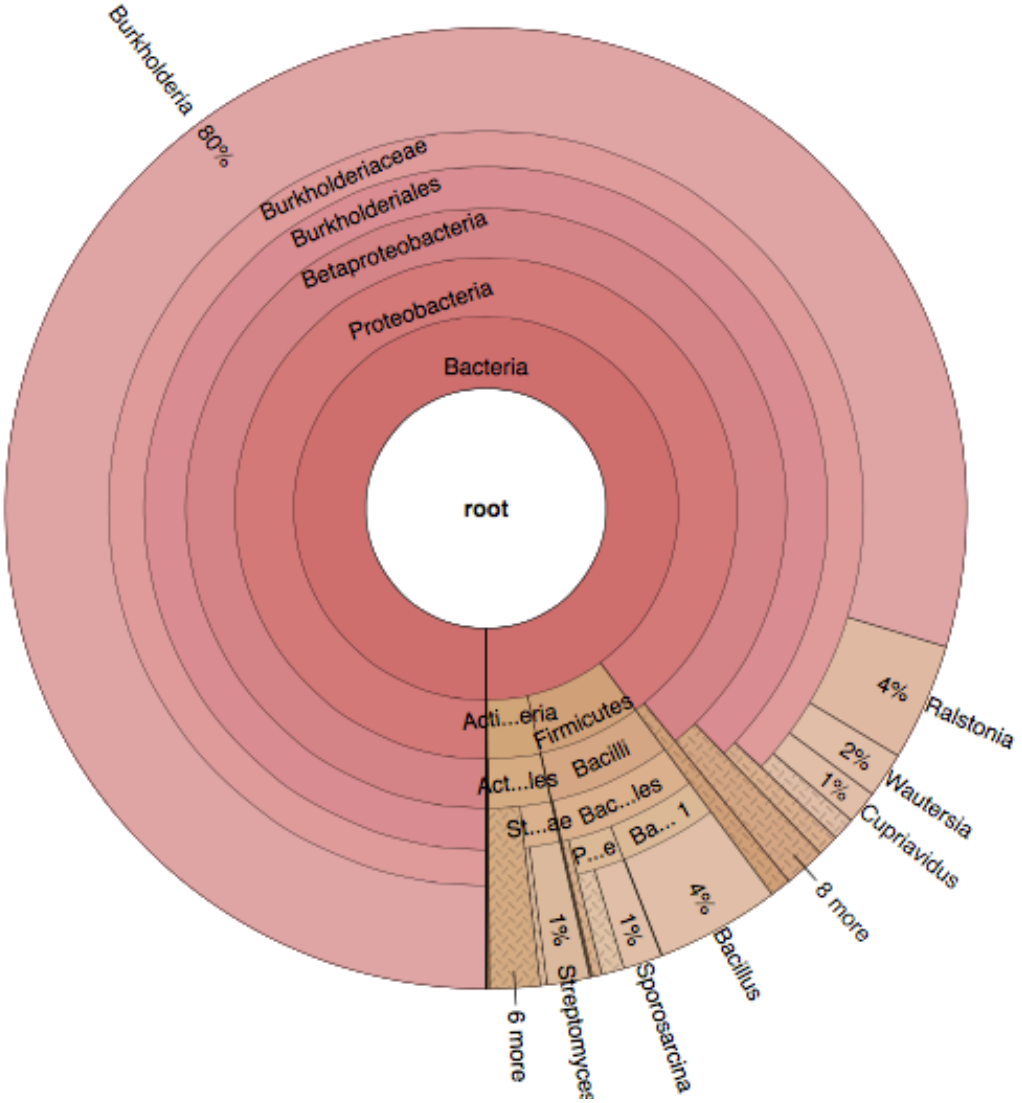

Figure 22 Microbiome of termite hindgut from the Appomattox River cultured in selective cellulose media from the phylum to the genus level

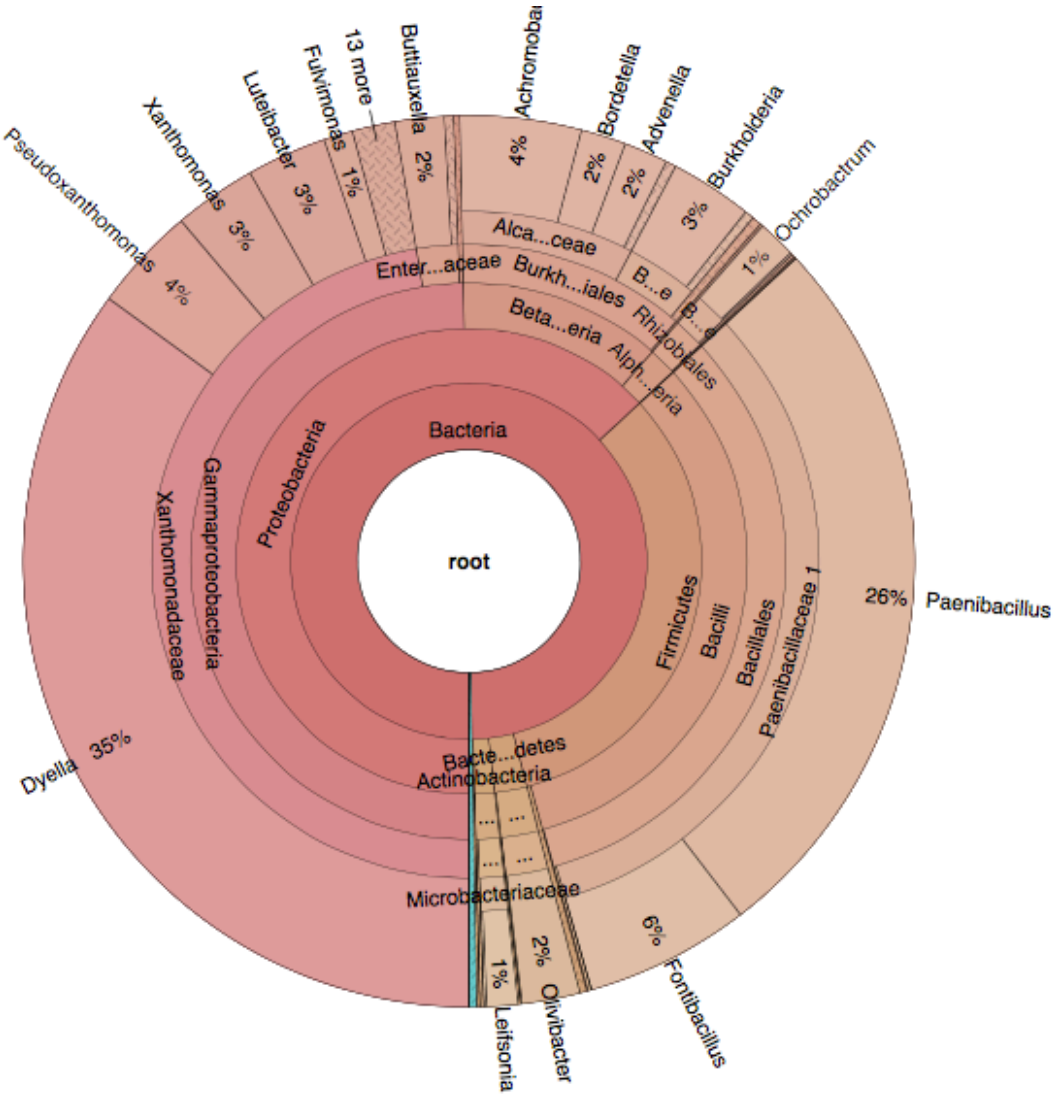

Figure 23 Microbiome of termite hindgut from the East Woods of VSU campus cultured in selective cellulose media from the phylum to the genus level

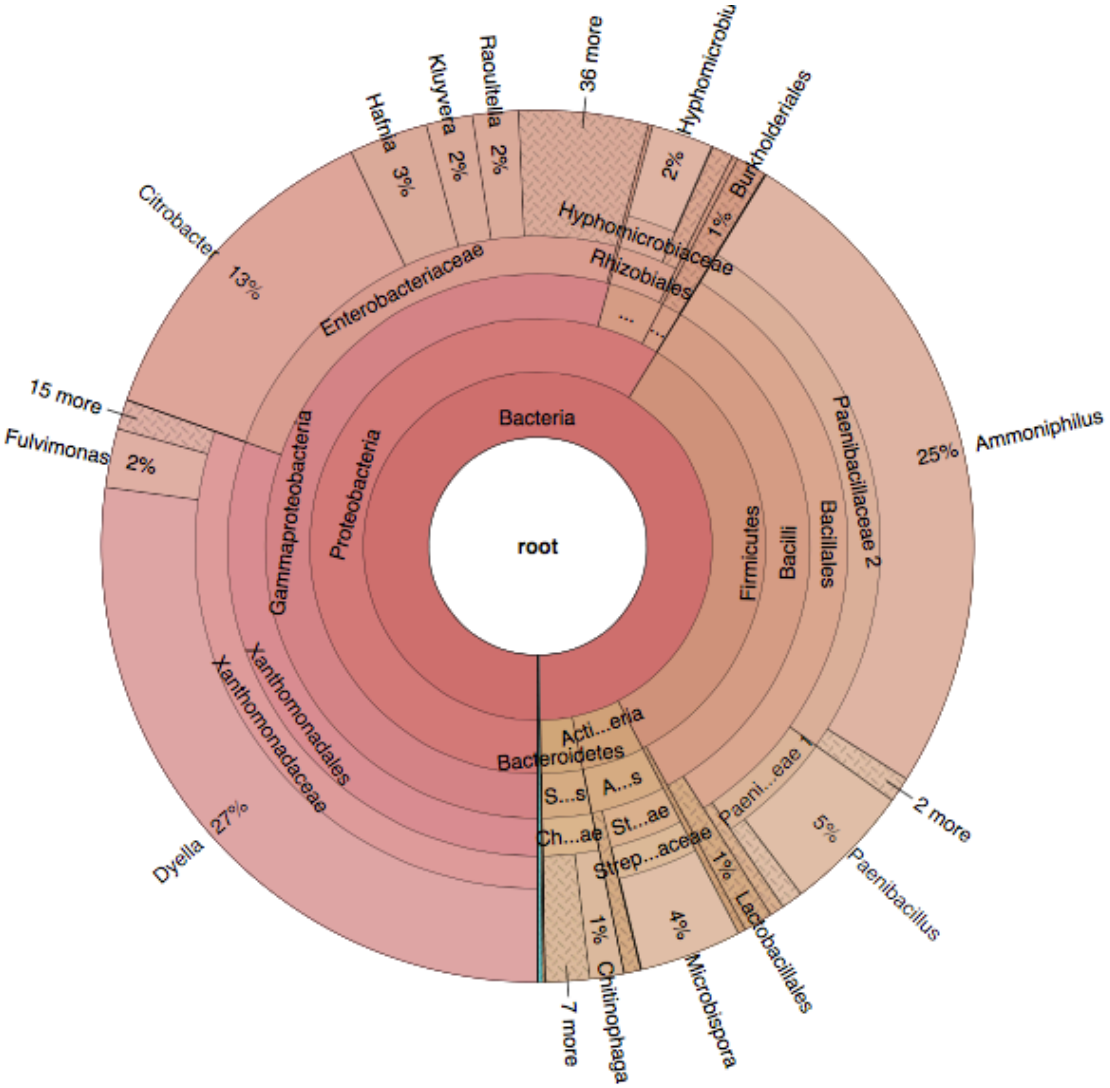

Figure 24 Microbiome of soil sample from the East Woods of VSU cultured in selective cellulose media from the phylum to the genus level

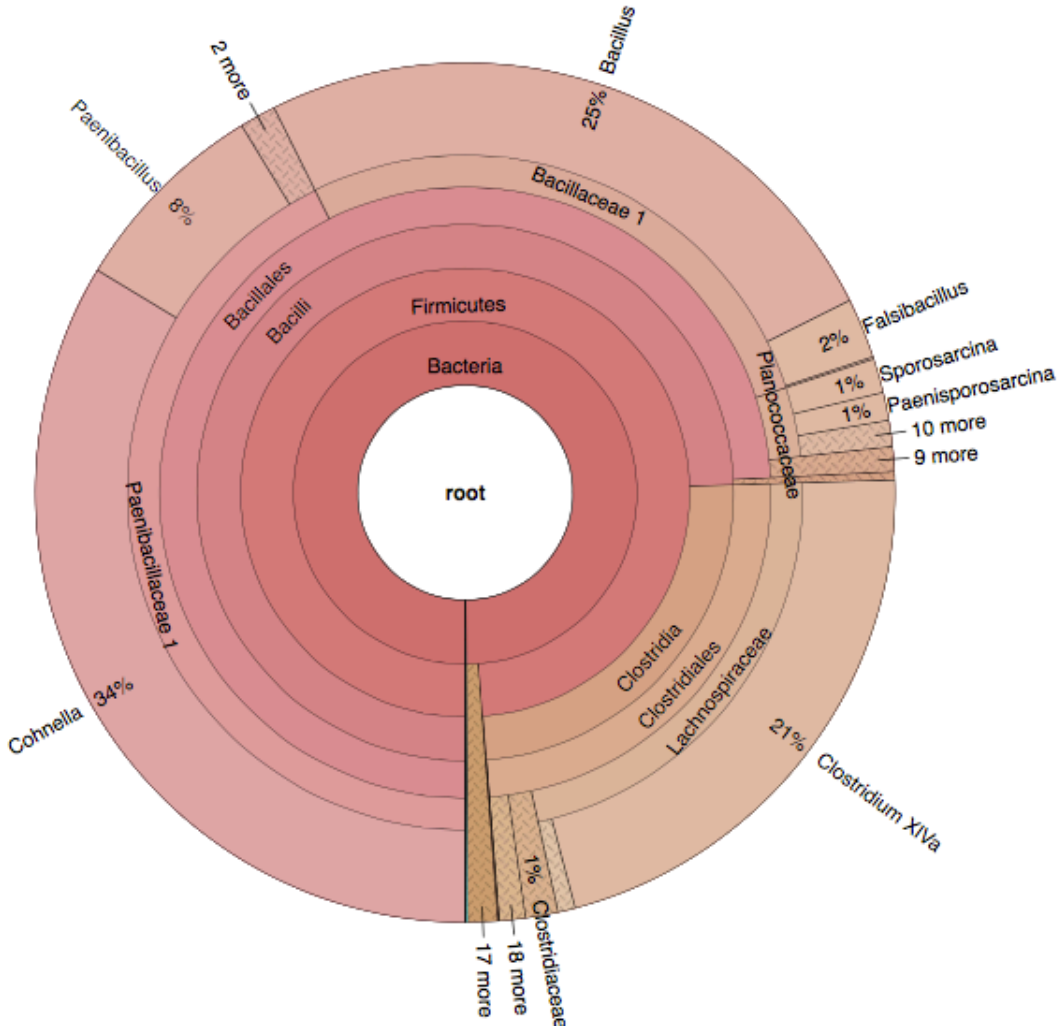

Figure 25 Microbiome of decaying leaf sample #2 cultured in selective cellulose culture from the phylum to the genus level

Table 2 Measurement of cell concentration at optical density 600 nanometers

Cell Measurement at OD 600		
ID	Absorbance (at 600nm)	Concentration (cells/ml)
C1	0.344	1.72E+08
C2	0.167	8.35E+07
C3	0.156	7.79E+07
C4	0.521	2.60E+08
C6	0.303	1.52E+08
C7	0.517	2.58E+08
C8	0.476	2.38E+08
C9	0.533	2.67E+08
C10	**0.55**	**2.75E+08**

Table 3 Total number of nucleotide sequence reads per sample

Sample ID	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**Mean**
Sequence Reads	316,017	180,427	500,535	237,322	273,167	340,728	334,011	217,392	176,065	203,676	277,934

Table 4 Cellulose Standards Measurement

Concentration (mg/ml)	Absorbance
5	0.004
10	0.01
25	0.073
50	0.118
75	0.196

Table 5 Cellulose Measurement at OD630

Identification	**Absorbance**
C1	0.008
C2	0.008
C3	0.01
C4	0.012
C5	0.011
C6	0.007
C7	0.014
C8	0.009
C9	0.007
C10	0.01

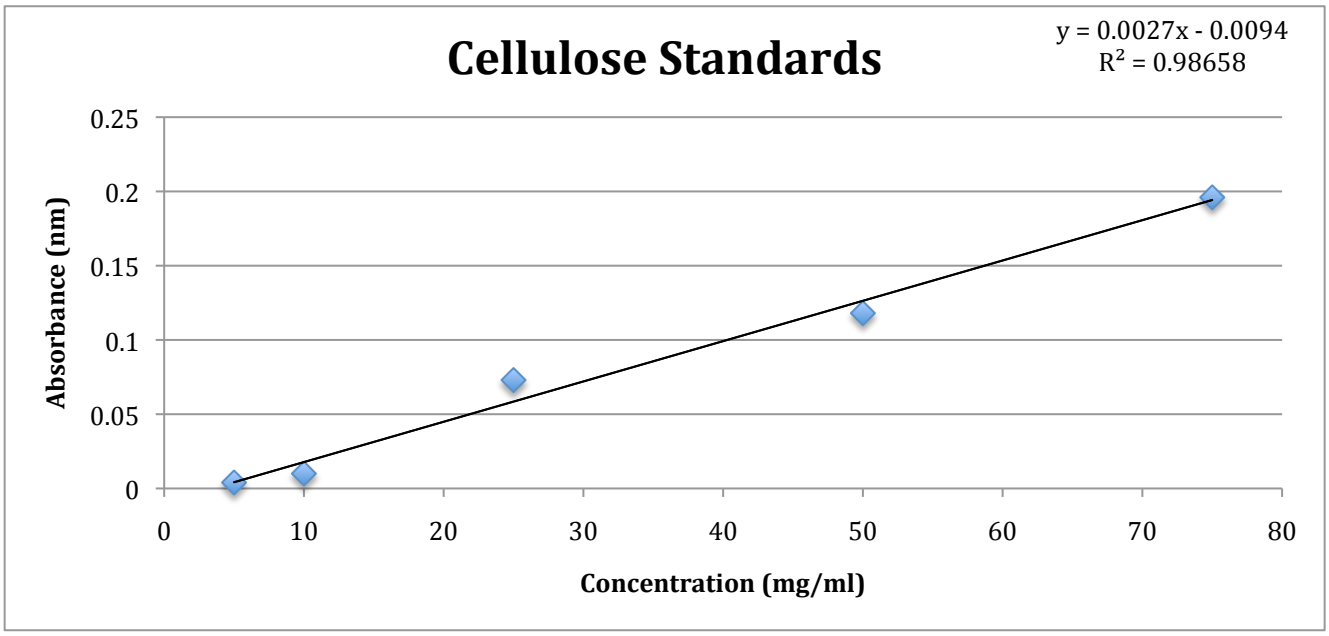

Figure 26 Standard curve for cellulose estimation

Table 6 Glucose Standards

Concentration	Absorbance (nm)
50	0
100	0.003
150	0.004
200	0.007
250	0.009
300	0.010
350	0.015
400	0.019
450	0.026
500	0.033

Table 7 Glucose Measurements at OD 510

Sample ID	Absorbance (nm)
C1	0.149
C2	0.152
C3	0.15
C4	0.148
C6	0.159
C7	0.154
C8	0.154
C9	0.153
C10	0.165

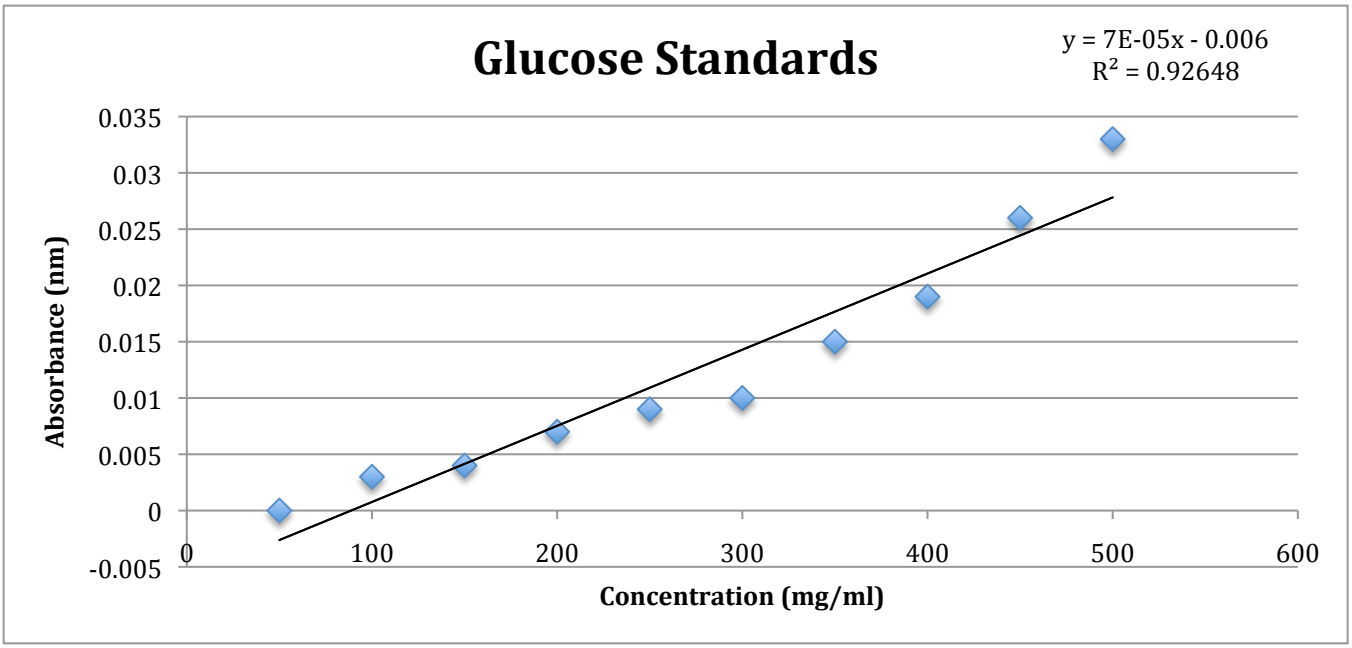

Figure 27 Standard curve for glucose estimation

Table 8 Summary of Biochemical Analysis

ID # (Using 5-75 Standards)	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
ID Description	Sheep #1 .22	Sheep #2 .22	Sheep #1 Wash off	Sheep#2 Wash off	Sheep #1 Unfiltered	Plant Fungi	Soil #3	Termite C2 APPOX.	Sheep #2 Unfiltered	EW Termite C1
Absorbance	0.008	0.008	0.01	0.012	0.011	0.007	0.014	0.009	0.007	0.01
Mass of Initial Cellulose (mg)	40	40	40	40	40	40	40	40	40	40
Concentration (ug/ml)	6.763	6.763	7.489	8.216	7.853	6.399	8.943	7.126	6.399	7.489
Mass of Remaining Cellulose (mg)	13.525	13.525	14.979	16.433	15.706	12.799	17.886	14.252	12.799	14.979
% of Cellulose Remaining	33.81%	33.81%	37.45%	41.08%	39.26%	32.00%	44.72%	35.63%	32.00%	37.45%
% of Cellulose Used	66.19%	66.19%	62.55%	58.92%	60.74%	68.00%	55.28%	64.37%	68.00%	62.55%
% of Glucose relative to the original total mass	17.86%	18.20%	17.98%	17.75%	NA	19%	18.43%	18.43%	18.32%	19.69%
% of Cellulose used for cell growth as Glucose	48.32%	47.98%	44.58%	41.17%	NA	49%	36.85%	45.94%	49.69%	42.86%
% of Glucose used for cell growth	73.01%	72.50%	71.26%	69.88%	NA	72.06%	66.66%	71.36%	73.06%	68.53%
% of Glucose Remaining	26.99%	27.50%	28.74%	30.12%	NA	27.94%	33.34%	28.64%	26.94%	31.47%

Table 9: Comprehensive listing of cellulolytic bacteria from selective cellulose cultures

Tax-id	Root rank	Domain	Phylum	Class	Order	Family	Genus
1633	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Escherichia/Shigel
1658	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Serrat
1639	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Leclerc
1656	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Salmonel
1631	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Enterobact
2953	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Lelliott
1626	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Citrobact
1623	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Buttiauxel
1628	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Cronobact
1653	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Rahnel
1647	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pectobacteriu
1666	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Yersin
1654	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Raoultel
1646	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Pantoe
1620	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Brenner
1657	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Samson
1644	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Morganel
1636	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Hafn
1618	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unclass_Enterobacter cea
1815	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Vibrionales	Vibrionaceae	unclass_Vibrionacea
1839	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Dyel
1841	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadals	Xanthomonadaceae	Fulvimona
1835	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	unclass_Xanthomonac cea
1784	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Serper

1781	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomona
1773	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	unclass_Pseudomonac cea
1763	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	unclass_Pseudomonadales	unclass_Pseudomonac le
1558	Root	Bacteria	Proteobacteria	Gammaproteobacteria	Alteromonadales	Shewanellaceae	Shewanel
1502	Root	Bacteria	Proteobacteria	Gammaproteobacteria	unclass_Gammaprot eobacteria	unclass_Gammaproteobacteria	unclass_Gammaprote bacter
1408	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Bilophi
1411	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	Desulfovibri
1408	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionacea	unclass_Desulfovibric acea
1396	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	unclass_Desulfovibrionales	unclass_Desulfovibric ale
1360	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Bdellovibrionales	Bdellovibrionaceae	Vampirovibri
1469	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	Desulfoglaeb
1468	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	unclass_Syntrophoba eracea
1462	Root	Bacteria	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	unclass_Syntrophobacterales	unclass_Sybacteracea
1227	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Pelomona
1207	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Acidovora
1249	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Oxalobacter
1241	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	unclass_Oxalobactera ea
1256	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	Aquabacterium
1196	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholder
1256	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_incertae_sedis	unclass_Burkholderia s_incertae_sed
1207	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	unclass_Comamonada ea
1196	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	unclass_Burkholderia ea
1314	Root	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	Stenoxybact
1287	Root	Bacteria	Proteobacteria	Betaproteobacteria	Neisseriales	Neisseriaceae	unclass_Neisseriacea

1343	Root	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Propionivibri
1348	Root	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	Sulfuritale
1332	Root	Bacteria	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	unclass_Rhodocyclace
1177	Root	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	unclass_Burkholderiales	unclass_Burkholderia
1353	Root	Bacteria	Proteobacteria	Deltaproteobacteria	unclass_Deltaproteobacteria	unclass_Deltaproteobacteria	unclass_Deltaproteoba ter
1176	Root	Bacteria	Proteobacteria	Betaproteobacteria	unclass_Betaproteobacteria	unclass_Betaproteobacteria	unclass_Betaproteoba er
1074	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	Acidisom
1071	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	unclass_Acetobactera ea
1125	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Telmatospirillu
885	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinckiaceae	Methylovirgu
892	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobiu
887	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	unclass_Bradyrhizob cea
918	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae	Hyphomicrobiu
929	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacteriu
1161	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobiu
1159	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	unclass_Sphingomona acea
1153	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	unclass_Erythrobacte cea
1070	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	unclass_Rhodospirillales	unclass_Rhodospirill e
871	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	unclass_Rhizobiales	unclass_Rhizobiale
1152	Root	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	unclass_Sphingomonadales	unclass_Sphingomona ale
843	Root	Bacteria	Proteobacteria	Alphaproteobacteria	unclass_Alphaproteobacteria	unclass_Proteobacteria	unclass_Alphaproteob cter
842	Root	Bacteria	Proteobacteria	unclass_Proteobacteria	unclass_Proteobacteria	unclass_Proteobacteria	unclass_Proteobacter

2513	Root	Bacteria	Firmicutes	Negativicutes	Selenomonadalesnadales	Acidaminococcaceae	Phascolarctobacteriu
2725	Root	Bacteria	Firmicutes	Negativicutes	Selenomonadales	Acidaminococcaceae	unclass_Acidaminocc cacea
2724	Root	Bacteria	Firmicutes	Negativicutes	Selenomonadales	unclass_Selenomonadales	unclass_Selenomonac lesss_Selenomonadale
2679	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Clostridium XI
2676	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVa
2626	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Blautia
2677	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Clostridium XlVb
2449	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Roseburia
2446	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	unclass_Lachnospirac ae
2480	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	unclass_Pept ostreptococcaceae
2615	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Butyricicoccus
2494	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Sporobacter
2486	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Acetivibrio
2493	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Papillibacter
2490	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Ethanoligenens
2682	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Clostridium IV
2491	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Anaerotruncus
2489	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Acetanaerobacterium
2639	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Oscillibacter
2681	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Clostridium III
2485	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Faecalibacterium
2644	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Hydrogenoanaerobact rium
2485	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	unclass_Ruminococca eae
2434	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XIII	Anaerovorax
2434	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiales_Incertae Sedis XIII	unclass_Clostridiales_ ncertae Sedis XIII

2472	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae 1	Dehalobacter
2474	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae 1	Desulfosporosinus
2471	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae 1	Syntrophobotulus
2471	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Peptococcaceae 1	unclass_Peptococcaceae 1
2399	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	Anaerofustis
2396	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Eubacteriaceae	unclass_Eubacteriacea
2378	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	unclass_Clostridiaceae 1
2500	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	Syntrophomonas
2498	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	Syntrophomonadaceae	unclass_Syntrophomonadaceae
2377	Root	Bacteria	Firmicutes	Clostridia	Clostridiales	unclass_Clostridiales	unclass_Clostridiales
2298	Root	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae 1	Paenibacillus
2597	Root	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae 1	Fontibacillus
2268	Root	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Fontibacillus
2309	Root	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	unclass_Planococcaceae
2298	Root	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae 1	unclass_Paenibacillaceae 1
2262	Root	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae 1	unclass_Bacillales
2370	Root	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Oenococcu
2368	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Leuconostocaceae	Weissel
2369	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Leuconostocaceae	Leuconosto
2373	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Streptococcaceae	Lactococcu
2374	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Streptococcaceae	Lactovu
2362	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Enterococcaceae	Enterococcu
2358	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Enterococcaceae	Pilibact
2368	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Leuconostocaceae	unclass_Leuconostocaea
2358	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Enterococcaceae	unclass_Enterococcaca
2372	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Streptococcaceae	unclass_Streptococcac

2343	Root	Bacteria	Firmicutes	Bacilli	Lactobacillale	Carnobacteriaceae	unclass_Carnobacteria
2334	Root	Bacteria	Firmicutes	Bacilli	Lactobacillales	unclass_Lactobacillales	unclass_Lactobacillale
2671	Root	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichals	Erysipelotrichaceae	Clostridium XVI
2561	Root	Bacteria	Firmicutes	Erysipelotrichia	Erysipelotrichales	Erysipelotrichaceae	unclass_Erysipelotrich
2376	Root	Bacteria	Firmicutes	Clostridia	unclass_Clostridia	unclass_Clostridia	unclass_Clostrid
2261	Root	Bacteria	Firmicutes	Bacilli	unclass_Bacilli	unclass_Bacilli	unclass_Bacilli
2260	Root	Bacteria	Firmicutes	unclass_Firmicutes	unclass_Firmicutes	unclass_Firmicutes	unclass_Firmicute
108	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Cellulomona
205	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Promicromonosporaceae	unclass_Promicromon
172	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	Leifson
155	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Microbacteriaceae	unclass_Microbacteria
151	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Intrasporangiaceae	Tetrasphae
279	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Tessaracocc
275	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionicicel
265	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionnibacteriaceae	unclass_Propionibact
96	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	unclass_Actinomycetales	unclass_Actinomycet
36	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	unclass_Actinomycetales	unclass_Actinomycet
255	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	unclass_Actinomycetales	unclass_Actinomycet
18	Root	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	unclass_Actinomycetales	unclass_Actinomycet
375	Root	Bacteria	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	Gordonibact
366	Root	Bacteria	Actinobacteria	Actinobacteria	Coriobacteriales	Coriobacteriaceae	unclass_Coriobacteria
17	Root	Bacteria	Actinobacteria	Actinobacteria	unclass_Actinobacteria	unclass_Actinobacteria	unclass_Actinobacter

15	Root	Bacteria	Actinobacteria	Actinobacteria	Acidimicrobiales	Acidimicrobineae_incertae	Aciditerrimona
4	Root	Bacteria	Actinobacteria	Actinobacteria	unclass_Actinobacteria	unclass_Actinobacteria	unclass_Actinobacter
439	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Dysgonomona
446	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Proteiniphilu
447	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Tannerel
443	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Parabacteroid
442	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Paludibact
437	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Barnesiel
437	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Porphyromonad	Unclass_Porphyromo adacea
430	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroid
2957	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Marinilabiliaceae	Mangroviflexu
2846	Root	Bacteria	Bacteroidetes	Bacteroidia	Bateroidales	Marinilabiliaceae	Vnilabiliacea
454	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	Alistip
454	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Rikenellaceae	unclass_Rikenellacea
451	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	Prevotellaceae	Prevotel
425	Root	Bacteria	Bacteroidetes	Bacteroidia	Bacteroidales	unclass_Bacteroidales	unclass_Bacteroidal
611	Root	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagaceae	Menisc
548	Root	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Soonwoo
481	Root	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Chryseobacteriu
471	Root	Bacteria	Bacteroidetes	Flavobacteriia	Flacobacteriales	Flavobacteriaceae	unclass_Flavobacteria ea
460	Root	Bacteria	Bacteroidetes	Flavobacteriia	Flavoobacteriales	unclass_Flavobacteriales	unclass_Flavobacteri e
423	Root	Bacteria	Bacteroidetes	unclass_Bacteroidetes	unclass_Bacteroidetes	unclass_Bacteroidetes	unclass_Bacteroidet
1903	Root	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Treponem
2868	Root	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Sphaerochae
1902	Root	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	Spirochae
1896	Root	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	Spirochaetaceae	unclass_Spirochaetace
1886	Root	Bacteria	Spirochaetes	Spirochaetia	Spirochaetales	unclass_Spirochaetales	unclasss_Spirochaeta

2962	Root	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	Pirellu
828	Root	Bacteria	Planctomycetes	Planctomycetia	Planctomycetales	Planctomycetaceae	unclass_Planctomyce cea
826	Root	Bacteria	Planctomycetes	Planctomycetia	unclass_Planctomyc etia	unclass_Planctomycetia	unclass_Planctomyce
2771	Root	Bacteria	Elusimicrobia	Endomicrobia	unclasss_Endomicro bia	unclass_Endomicrobia	Candidatu Endomicrobiu
783	Root	Bacteria	Elusimicrobia	Elusimicrobia	Elusimicrobiales	Elusimicrobiaceae	Elusimicrobiu
2147	Root	Bacteria	Candidatus Saccharibacteria	unclass_Candidatus Saccharibacteria	unclass_Candidatus Saccharibacterididat us Saccharibacteria	unclass_Candidatus Saccharibacteria	Saccharibacteria_gen a_incertae_sed
2173	Root	Bacteria	Verrucomicrobia	Opitutae	Punicelcoccales	Puniceicoccaceae	Coraliomargari
2172	Root	Bacteria	Verrucomicrobia	Opitutae	Punicelcoccales	Puniceicoccaceae	unclass_Puniceicocc ea
2169	Root	Bacteria	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitut
2166	Root	Bacteria	Verrucomicrobia	Opitutae	Opitutales	unclass_Opitutae	unclass_Opitut
2177	Root	Bacteria	Verrucomicrobia	Spartobacteria un	Opitutales	unclass_Spartobacteria	Spartobacteria_genera incertae_sed
2911	Root	Bacteria	Verrucomicrobia	Verrucomicrobiae	Opitutales	Verrucomicrobiaceae	Roseimicrobiu
2188	Root	Bacteria	Verrucomicrobia	Verrucomicrobiae	Opitutales	Verrucomicrobiaceae	unclass_Verrucomicro iacea
2185	Root	Bacteria	Verrucomicrobia	Verrucomicrobiae	Opitutales	unclass_Verrucomicrobiales	unclass_Verrucomicro iale
2177	Root	Bacteria	Verrucomicrobia	Spartobacteria	Opitutales	unclass_Spartobacteria	unclass_Spartobacter
2257	Root	Bacteria	Acidobacteria	Acidobacteria_Gp1	Opitutales	unclass_Acidobacteria_Gp1	Terriglobu
2205	Root	Bacteria	Acidobacteria	Acidobacteria_Gp1	Opitutales	unclass_Acidobacteria_Gp1	Gp
2256	Root	Bacteria	Acidobacteria	Acidobacteria_Gp1	Opitutales	unclass_Acidobacteria_Gp1	Granulicel
2205	Root	Bacteria	Acidobacteria	Acidobacteria_Gp1	Opitutales	unclass_Acidobacteria_Gp1	unclass_Acidobacteria Gp
2209	Root	Bacteria	Acidobacteria	Acidobacteria_Gp3	Opitutales	unclass_Acidobacteria_Gp3	Gp
2196	Root	Bacteria	Acidobacteria	unclass_Acidobacteria	Opitutales	unclass_Acidobacteria	unclass_Acidobacter
755	Root	Bacteria	Deferribacteres	Deferribacteres	Opitutales	Deferribacteraceae	Mucispirillu
750	Root	Bacteria	Deferribacteres	Deferribacteres	Opitutales	Deferribacteraceae	unclass_Deferribacte

								cea
2165	Root	Bacteria	Verrucomicrobia	unclass_Verrucomicrobia	Opitutales	unclass_Verrucomicrobia	unclass_Verrucomicro	
1914	Root	Bacteria	Synergistetes	Synergistia	Opitutales	Syneraegistace	Cloacibacillu	
1920	Root	Bacteria	Synergistetes	Synergistia	Opitutales	Thermovirga	Thermovirg	
1910	Root	Bacteria	Synergistetes	Synergistia	Opitutales	Aminiphilus	Aminiphilu	
1910	Root	Bacteria	Synergistetes	Synergistia	Opitutales	unclass_Synergistaceae		
3	Root	Bacteria	Actinobacteria	unclass_Actinobacteria	Opitutales	unclass_Actinobacteria	unclass_Actinobacter	
2145	Root	Bacteria	SR1 unclass_SR1	unclass_SR1	Opitutales	SR1_genera_incertae_sedis	uncla	
816	Root	Bacteria	Lentisphaerae	Lentisphaeria	Opitutales	Victivallaceae	Victivall	
1944	Root	Bacteria	Tenericutes	Mollicutes	Opitutales	Mycoplasmaticeae	Ueraplas	
1941	Root	Bacteria	Tenericutes	Mollicutes	Opitutales	Mycoplasmataceae	unclass_Mycoplasma	cea
825	Root	Bacteria	Planctomycetes	unclass_planctomycetes	Opitutales	unclass_planctomycetes	unclass_planctomyce	
2751	Root	Bacteria	unclass_candidate division ZB3	unclass_candidate division ZB3	Opitutales	unclass_candidate division ZB3	unclass_candida	division ZB
2	Root	Bacteria	unclass_Bacteria	unclass_Bacteria	Opitutales	unclass_Bacteria	unclass_Bacter	

CHAPTER IV

DISCUSSION AND CONCLUSION

Using the 16s rRNA gene as a marker and selective culture with cellulose, this study identified the cellulose-degrading bacteria from soil, sheep rumen, termite hindgut, twig, and decaying plant samples. To be considered a candidate for biofuel production, bacteria must have the ability to degrade the tough structural component of green plants, cellulose. If the bacteria were able to grow and thrive in the presence of cellulose, it can potentially be used to degrade it. Each sample after the selective culture showed a diverse array of bacteria, but the majority of the bacteria belong to three phyla: Proteobacteria, Firmicutes, and Bacteroidetes. A comprehensive listing of cellulolytic bacteria from selective cellulose cultures is outlined in Table 9.

Comparison of the nonselective and selective cultures displayed different levels of diversity and microbial compositions as expected. For example, the nonselective culture of the termite hindgut bacterial community (C1 1:100 dilution) contains greater diversity of bacteria than the selective culture. The composition of the bacterial community has also changed after the cellulose selective culture. For the termite sample collected in the East Woods on VSU campus, Firmicutes' share decreased while Proteobacteria increased after the cellulose selective culture. A further examination at the class level suggests lower taxonomic level comparison may be more meaningful. For example, within both Firmicutes and Proteobacteria, the dominant class completely changed after the cellulose selective culture. The comparison of the two termite collections after selective cultures

reveals a similar composition of cellulose-degrading bacteria at the phylum level. Analysis at the genus level, however, shows the two termite colonies differ significantly in the diversity of cellulose-degrading bacteria. 80% of bacteria from cellulose selective culture of the Appomattox River termite colony belong to the genus Burkholderia. Examination of the VSU East Woods termite sample shows two dominant groups of bacteria at the genus level: Dyella (35%) and Paenibacillus (26%). However, the highest level of similarity was found between the selective cultures of the soil and termite collections from the same site. These results suggest that the cellulose-degrading microbial composition of termites may be determined by the soil microenvironment they live in. This may be understandable given that termites lives in soil and use soil, together with their own saliva and feces, to construct their nests.

A previous metagenomic analysis of soil composition from Phatthalung, Thiland reveals 5 cellulose-degrading bacteria identified to be derived from the genus Cellulomonas (Sangkhara 2011). Another study has also identified cellulose-degrading bacteria in soil as species in the genera Micrococcus, Bacillus, Pseudomonas, Xanthomonas and Brucella (Behera 2014). These genera of bacteria belong to the plylum of either Firmicutes or Proteobacteria, both of which have been shown to be the major groups (constituting at least 1% of the community) in this study of the soil sample after cellulose selective culture. However, at the genus level, none of the above genera constitutes a significant proportion of cellulose-degrading bacteria in our soil sample. Instead, our metagenomic study reveals different genera to be the major groups of cellulose-degrading bacteria, with Dyella (27%), Ammoniphilus (25%), Citrobacter (13%) being the three largest groups.

Since the decaying leaf was collected on the forest floor, we expected a near identical microbial community to that in the soil. Further analysis revealed a completely different microbiome where there were no significant commonalities between the two types of samples at the genus level. The most abundant genera of bacteria represented in the decaying leaf sample were 34% Cohnella, 25% Bacillus, and 21% Clostridia, which is completely different from that in the soil as described above. Interestingly, the two leaf samples, which originally came from different areas of the same leaf, contain very different compositions of bacteria. One leaf collection was cut into pieces and cultured on separate LB plates, then cultured in selective cellulose media. Initial analysis of the physical state of the leaf revealed portions that were significantly more decayed than other parts of the leaf. This visualization would explain why different parts of the same leaf contain very different microbial compositions.

The significant difference in the microbiome of sheep rumen #1 and #2 is intriguing considering both samples were collected from the same facility. Analysis at the genus level of the unfiltered sheep rumen #1 after cellulose selective culture reveals a microbiome composition of 28% Microbacterium, 21% Cohnella, and 19% Lactococcus with at least 92% confidence. In striking contrast, Luteimonas represent 92% of the sheep rumen #2 sample after the selective culture at the genus level. What is not clear about the two individuals, however, is their origin, diet, breed, and physical environment they were housed in before arriving to the collection facility. Previous studies conducted on the sheep rumen have characterized the cellulose degrading bacteria into two groups based on predominant cellulose degrading potential: Bacteroides and Ruminococcus (Russell 2009). Both of these groups however represented insignificant percentages of our rumen

samples. This demonstrates the value of metagenomic study in quantifying the relative frequency of different groups of bacteria.

Comparison of environmental microbiomes and host microbiomes show a difference in the composition and overall diversity among sample collections. Termite hindgut collections contained more cellulolytic bacteria than the leaf and twig samples. Proteobacteria exclusively dominated leaf #1 and twig samples, whereas the Termite Hindgut is only partially comprised of Proteobacteria. Bacteria characterized in the hindgut are necessary for cellulose digestion within the termites diet. Leaf #2 was largely decayed and contained different bacteria than that of the Termite and other environment samples. The cellulose-degrading bacteria found in environmental samples are based on the physical conditional of the sample, the extent of decay, and the overall breakdown of cellulose in plants. Surprisingly, bacteria typically associated with the gut of animal host on the twig samples. Since the twig was found on the forest floor, this is likely due to the twig coming in contact with body fluids or byproducts from an animal host. Cellulose-degrading bacteria from sheep collections were not only significantly different from each other, but also different from the environmental samples and Termite samples. These samples have great diversity compared to environmental samples, but less abundance among these cellulose-degrading bacteria. Comparison of host and environmental cellulose-degrading bacteria vary significantly based on the physical condition and host where the samples were collected.

As we face the potential depletion of fossil fuels, we must become proactive in the exploration of renewable energy sources to fuel our energy demands. With the use of fossil fuels, we will also continue to endure the harmful effects including water and air

pollution, medical complications, and the inevitable global warming. The established corn-based bioethanol production is economically, environmentally, and socially too costly, while next-generation bioethanol production from the more abundant cellulosic plant materials offers much more cost-effective, environmentally friendly, and renewable alternative energy. Cellulose-degrading bacteria in three main phyla (Proteobacteria, Firmicutes, and Bacteroidetes) have great potential to be used in the cellulosic bioethanol production. The characterization of these cellulose-degrading bacteria at the genus level will help develop the next-generation bioethanol technology.

REFERENCES

1. Aurélien T., Scharf M.E., Marsha W., Xuguno Z., Monique C., Drion B., and Michael S. 2009. Parallel metatranscriptome analyses of host and symbiont gene expression in the gut of the termite reticulitermes flavipes. *Biotechnology for Biofuels* 18: 1-19.

2. Bayer E., Lamed R., and Himmel M. 2007. The potential of cellulases and cellulosomes for cellulosic waste management. *Current Opinion Biotechnology* 18: 237–245.

3. Bhat M. 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances* 18: 355–383.

4. Hendricks C., Doyle J., and Hugley B. 1995. A new solid medium for enumerating cellulose-utilizing bacteria in soil. *Applied and Environmental Microbiology* 61: 2016–2019.

5. Correa J. Digestive system of goats. 2007. Alabama, Alabama Cooperative Extension System.

6. DOE Joint Genome Institute. 2007. Termite guts may yield novel enzymes for better biofuel production. ScienceDaily: http://www.sciendaily.com. Extracted on September 25, 2014.

7. The World Bank. 2015. Fossil fuel energy consumption (% of Total). http://data.worldbank.org/indicator/EG.USE.COMM.FO.ZS. Extracted on April 4, 2014.

8. Fujita A., Masaru H., Tetsuo A., Yoshinobu H., Gaku A., Gaku T., and Hirofumi W. 2010. Details of the digestive system in the midgut of Coptotermes formosanus Shiraki. *Journal of Wood Science* 56: 222-226.

9. Geddes C., Claudia C., Ismael N., and Lonnie I. 2011. Advances in ethanol production. *Current Opinion in Biotechnology* 22: 312-319.

10. Geng A., Gen Z., Xing Y., Qianfu W., Jun Z., Fanghua L., Baoli Z., and Zhihua Z. 2012. Expression and characterization of a novel metagenome-derived cellulase exo2b and its application to improve cellulase activity in trichoderma reesei. *Applied Microbiology and Biotechnology* 96: 951-62.

11. Handelsman J., Rondon M., Brady S., Clardy J., and Goodman R. 1998. Molecular biology provides access to the chemistry of unknown soil microbes: a new frontier for natural products. *Chemistry Biology* 5: R245–R249.

12. Haichar F., Wafa A., Richard C., Thierry H., Christine M., Marie-France M., Christophe M., Lionel R., Jérôme B., and Odile B. 2007. Identification of cellulolytic bacteria in soil by stable isotope probing. *Environmental Microbiology* 9: 625-34.

13. Hughes J., Hellmann J., Ricketts T., and Bohannan B. 2014. Counting the uncountable: statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology* 67: 4399-4406.

14. Imshenetsky A. 1968. Decomposition of cellulose in the soil, In: T. R. G. Gray and D. Parkinson, *The Ecology of Soil Bacteria.* Toronto: University of Toronto Press.

15. Janda M. and Shanron A. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of Clinical Microbiology* 45: 2761-764.

16. Kang Y., Kim J., Seol Y., Lee C., and Cho T. 2009. Characterization of conjugative plasmids carrying antibiotic resistance genes encoding 16S rRNA methylase, extended-spectrum beta-lactamase, and/or plasmid-mediated AmpC beta-lactamase. *Microbiology* 47: 68-75.

17. Kersters K., Paul V., Monique G., Jean S., Peter V., and Erko S. 2006. Introduction to the proteobacteria. *Prokaryotes* 5: 3-37.

18. Kumar S., Singh S., Mishra I., and Adhikari D. 2009. Recent advances in production of bioethanol from lignocellulosic biomass. *Chemical Engineering & Technology* 32: 517-26.

19. Leschine B. 1995. Cellulose degradation in anaerobic environments. *Annual Review of Microbiology* 49: 399-426.

20. Liang C., Ederson J., David D., Randall J., James T., and Teri B. 2012. Soil microbial communities under model biofuel cropping systems in southern wisconsin, USA: impact of crop species and soil properties. *Applied Soil Ecology* 54: 24-31.

21. Li L., Frohlich J., Pfeiffer P., and Konig H**.** 2003. Termite gut symbiotic archaezoa are becoming living metabolic fossils. *American Society of Microbiology* 2**:** 1091-1098.

22. Mardis W. 2008. Next-generation DNA sequencing methods. *Annual Review of Genomics and Human Genetics* 9: 387-400.

23. Ni J., and Tokuda G. 2013. Lignocellulose-degrading enzymes from termites and their symbiotic microbiota. *Biotechnology Advances* 31: 838-850.

24. Ohkuma M. 2003. Termite symbiotic systems: efficient bio- recycling of lignocelluloses. *Applied Microbiology Biotechnology* 1: 1–9.

25. Pourramezan Z., Ghezelbash G., Romani B., Ziaei S., and Hedayatkhah A. 2012. Screening and identification of newly isolated cellulose-degrading bacteria from the gut of xylophagous termite microcerotermes diversus (Silvestri). *Microbiology* 81: 736-42.

26. Russell J., Muck R., and Weimer P. 2009. Quantitative analysis of cellulose degradation and growth of cellulolytic bacteria in the rumen. *FEMS Microbiology Ecology* 67: 183-97.

27. Sadasivam S. and Manickam.A. 1996. Biochemical methods, 2nd ed. New Delhi: New Age International.

28. Sangkhara K., Vangsirikul P., and Janthachat S. 2011. Isolation of novel cellulose from agricultural soil and application for ethanol production. *International Journal of Advanced Biotechnology and Research* 2: 230-239.

29. Sethi A., Slack J., Kovaleva E., Buchman G., and Scharf M. 2012. Lignin-associated metagene expression in a lignocellulose-digesting termite. *Insect Biochemistry and Molecular Biology* 43: 91-101.

30. Steele H. and Streit W. 2005. Metagenomics: advances in ecology and biotechnology. *FEMS Microbiology Letter* 247: 105-111.

31. Streit W. and Schmitz R. 2004. Metagenomics-the key to the uncultured microbes. *Current Opinion Microbiology* 7: 492-498.

32. Upadhyaya S., Anuroop M., Hemanta M., Anaya P., Anurag R., Barun P., and Bhabuk K. 2012. Isolation and characterization of cellulolytic bacteria from gut of termite. *Rentech Symposium Compendium* 1: 14-18.

33. Vetrovsky T. and Baldrian P. 2013. The variability of the 16S rRNA gene in bacterial genomes and its consequences for bacterial community analyses. *PLOS One 8*: e57923.

34. Lu W., Wang H., Nie Y., Wang Z., Huang D., Qiu X., and Chen J. 2004. Effect of inoculating flower stalks and vegetable waste with ligno-cellulolytic microorganisms on the composting process. *Journal of Environmental Science and Health* 39: 871– 887.

35. Yang B. and Wyman C. 2007. Pretreatment: The key to unlocking low-cost cellulosic ethanol. *Biofuels, Bioproducts & Biorefining* 2: 26-40.

36. Zheng Y., Zhongli P., and Ruihong Z. 2009. Overview of biomass pretreatment for cellulosic ethanol production. *International Journal of Agriculture and Biological Engineering* 2: 51-68.